CXCR4 ANTAGONISTS AND METHODS OF THEIR USE

CROSS REFERENCE TO RELATED APPLICATION

This application claims benefit of and priority to U.S. Provisional Patent Application No. 60/458,217 filed on March 27, 2003, which is incorporated by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

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BACKGROUND

1. Technical Field

The disclosure is generally directed to antagonists of chemokine receptors, particularly the CXCR4 receptor, and methods of their use, for example, in the treatment, prevention, or diagnosis of cancer.

2. Related Art

According to the American Cancer Society, approximately 1.3 million Americans are estimated to be diagnosed with invasive cancer in 2003. The National Cancer Institutes estimates that approximately 8.9 million Americans had a history of cancer in 2003, and approximate 1,500 cancer-related deaths per day are expected in 2003. Because of the staggering number of cancer-related deaths and new cases, new medicines and methods of treatment are needed. Although recent advances have increased our understanding of some of the mechanisms leading to cancer, effective treatments for cancer remain in high demand.

Cancer can be a fatal disease, in part, because cancer can spread or

metastasize throughout an organism. Metastasis plays a major role in the morbidity and mortality of breast cancer (Ford, K. et al. Breast cancer screening, diagnosis, and treatment. Dis. Mon., *45*: 333-405, 1999). Breast cancer metastasizes in a stereotypical pattern resulting in lesions found in the lymph node, lung, liver, and bone marrow. Generally, cancer cells lose differentiated properties, proper tissue compartmetalization, cell-cell attachment as well as obtain altered cell substratum attachment, altered cytoskeletal organization, cell locomotion, and the ability to survive at distant sites.

Treatments for invasive cancers such as breast cancer historically include surgery, radiation, anti-hormonal therapy, and chemotherapy. Although each therapy has some degree of success, the failure to achieve a cure in approximately 70% of patients is due to a primary lack of therapeutic effect on undetected or detected metastases and to acquired drug and hormonal resistance during therapy (Fidler, I. and Nicolson, G.L. Concepts and mechanisms of breast cancer metastases. In Bland, K.I., Copeland, E.M. (eds): The Breast. Philadelphia: WB Saunders, 1991, p 395).

Known therapies include those described in U.S. Patent No. 6,693,134 including naphthoic acid derivatives for treating diseases mediated by CXCR4.

U.S. Patent Application No. US2003220482 discloses a peptide fragment of vMIP-II that prevents the HIV-1 virus from interacting with the coreceptor CXCR4, thereby preventing viral infection of that cell.

Canadian Patent Application No. CA2245224 discloses peptides corresponding to the N-terminal 9 residues of stromal cell derived factor-1 (SDF-1) and have SDF-1 activity. Additionally, the patent application reports that SDF-1, 1-8, 1-9, 1-9 dimer and 1-17 induced intracellular calcium and chemotaxis in T lymphocytes and CEM cells, and

bound to CXC chemokine receptor 4 (CXCR4).

Canadian Patent Application No. CA2408319 discloses CXCR4 antagonists used to treat hematopoietic cells, such as progenitor or stem cells, to promote the rate of cellular multiplication, self-renewal, proliferation or expansion. The patent application further discloses that CXCR4 antagonists may be used therapeutically to stimulate hematopoietic stem/progenitor cell multiplication/self-renewal.

Canadian Patent Application No. CA2305787 discloses CXCR4 antagonists used therapeutically to stimulate hematopoietic cell multiplication, particularly progenitor or stem cell multiplication, in human diseases such as cancer.

WO0009152 discloses CXCR4 peptide antagonists corresponding to SDF-1 or comprising a partial sequence of SDF-1 for reducing interferon gamma production by T-cells, treatment of an autoimmune disease, treatment of multiple sclerosis, treatment of cancer, and inhibition of angiogenesis.

WO9947158 discloses peptide CXCR4 antagonists comprising a substantially purified peptide fragment, modified fragment, analogue or pharmacologically acceptable salt of SDF-1 for reducing interferon gamma production by T-cells, treatment of an autoimmune disease, treatment of multiple sclerosis, treatment of cancer, inhibition of angiogenesis.

Despite existing therapies for CXCR4 mediated pathologies, there remains a need for new and effective methods of treatment for CXCR4 related pathologies, including but not limited to cancer.

SUMMARY

Compositions and methods for the treatment or prevention of a chemokine-related or chemokine-receptor-related pathology are provided. In one aspect, the

pathology is cancer. It has been discovered that CXCR4 antagonists, in particular derivatives of T140 such as TN14003, TC14012, and TE14011 inhibit or reduce tumor metastasis in a host.

Another aspect of the disclosure provides CXCR4 polynucleotide antagonists. The disclosed CXCR4 polynucleotide antagonists include, but are not limited to, small interfering RNAs (siRNAs) which target CXCR4 mRNA. The polynucleotide antagonists can be administered "naked" to host or packaged to promote bioavailability and cell uptake. In still another embodiment, the polynucleotide antagonist is conjugated to a targeting substance such as folate.

Yet another aspect of the disclosure provides diagnostic compositions and methods for the detection, quantification, or identification of cancer cells and/or cancer cell metastases. The diagnostics include but are not limited to labeled CXCR4 antagonists.

Still another aspect provides pharmaceutical compositions for the treatment of cancer containing a therapeutic amount of a CXCR4 peptide antagonist. In one aspect, the CXCR4 peptide antagonist is not an antibody or antibody fragment.

Other compositions, methods, features, and advantages of the present disclosure will be or become apparent to one with skill in the art upon examination of the following drawings and detailed description. It is intended that all such additional compositions, methods, features, and advantages be included within this description, be within the scope of the present disclosure, and be protected by the accompanying claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A is an immunofluorescence micrograph of MDA-MB-231 cells using biotin-

labeled CXCR4 antagonist and streptavidin-conjugated rhodamine.

Fig. 1B provides Northern and Western Blots showing MDA-MB-231 cells had high levels of mRNA and protein for CXCR4 while MDA-435 cells did not.

Fig. 1C is an immunofluorescence micrograph of MDA-MB-231 cells and MDA-MB-435 cells using biotin-labeled CXCR4 antagonist and streptavidin-conjugated rhodamine that show MDA-MB-231 cells have higher levels of CXCR4 than MDA-MB-435 cells. Thus, biotinylated CXCR4 antagonists can quantitatively detect CXCR4 proteins on the cell surface.

Fig. 1D is a graph of flow cytometry data of MDA-MB-231 cells and MDA-MB-435 cells showing MDA-MB-435 cells had limited binding of the biotinylated CXCR4 antagonist. The biotinylated CXCR4 antagonist can be used to quantitatively detect CXCR4 protein by FACS analysis.

Fig. 1E is a panel of immunofluorescence micrographs of tissue sections using biotinylated CXCR4 antagonist.

Fig. 2 is a bar graph of matrigel invasion data of MDA-MB-231 cells treated with CXCR4 antagonist compared to anti-CXCR4 antibody from R&D company.

Fig. 3A is a panel of photographs showing no visible lung metastasis in the group treated with CXCR4 antagonist.

Fig. 3B is a bar graph of Real-Time RT-PCR of animal lungs treated with CXCR4 antagonist using primers specific for human CXCR4. The control peptide-treated animals could not gain weight due to lung metastasis.

Fig. 3C is a bar graph showing the average body weight was higher in antagonist treated animals compared to animals treated with control peptide.

Fig. 3D is a bar graph showing lung weight reflected the tumor burden of the

animal.

Fig. 4A is a bar graph showing that CXCR4 antagonist did not affect cell proliferation even at 10 nM concentration.

Fig. 4B are micrographs showing H&E staining of liver and kidney tissues from mice treated either with a PBS injection or CXCR4 antagonist.

Fig. 4C is a line graph showing that there were no discernable effects of CXCR4 antagonist on hemopoietic progenitor cell colony formation.

Fig. 4D is a line graph showing CXCR4 antagonist did not affect cell growth rate of normal human fibroblast 2091 cells even at 100 micromolar concentration.

Fig. 5A is an immunofluorescence micrograph of siRNA transfected MDA-MB-231 cells detected using the biotinylated CXCR4 antagonist and streptavidin-phycoerythrin (PE).

Fig. 5B is RT-PCT analysis of CXCR4 in siRNA transfected MDA-MB-231 cells showing siRNA1+2 effectively blocked the expression of CXCR4.

Fig. 5C is a Western blot of siRNA transfected MDA-MB-231 cells using anti-CXCR4 antibody Ab2 (1:1000). This also confims that siRNA1+2 effectively blocked CXCR4 expression.

Fig. 6A is photomicrograph showing H&E staining of the invasive MDA-MB-231 cells transfected with CXCR4 siRNAs. The invasion rate of MDA-MB-231 cells transfected with siRNA 1&2 was much less than that of MDA-MB-231 cells transfected with control siRNA.

Fig. 6B is a bar graph showing the invasion rates of MDA-MB-231 cells transfected with siRNA1&2, siRNA1, and siRNA2 relatively to the control are 6.9% (P=0.00028), 35.6% (P=0.00140), and 51.5%(P=0.00255) respectively.

Fig. 7A are photographs of the whole lungs of three mice from each group and H&E staining of these lung tissues show that the lungs from the treated group mice were normal while the lungs from the control group mice were filled with human tumor cells.

Fig. 7B is a bar graph showing RT-PCR of hHPRT results of lung samples from all animals in each group. Only two of six lungs from the siRNA of CXCR4 treated group mice expressed very low levels of detectable hHPRT.

DETAILED DESCRIPTION

The present disclosure may be understood more readily by reference to the following detailed description and the Examples included therein.

Before the present compounds, compositions and methods are disclosed and described, it is to be understood that this disclosure is not limited to specific pharmaceutical carriers, or to particular pharmaceutical formulations or administration regimens, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

1. Definitions

The term "organism" refers to any living entity comprised of at least one cell. A living organism can be as simple as, for example, a single eukaryotic cell or as complex as a mammal, including a human being.

The term "CXCR4 antagonist" means a substance including but not limited to a polypeptide, polynucleotide, inhibitory polynucleotide, or siRNA, that interferes or inhibits the biological activity of the CXCR4 receptor including, but not limited to, the binding of a ligand to the receptor. Exemplary CXCR4

antagonists include, but are not limited to TN14003, TC14012, and TE14011, and siRNAs directed to the CXCR4 receptor.

The term "CXCR4 peptide antagonist" means a polypeptide that specifically binds to CXCR4, particularly polypeptides that are not an antibody. Representative CXCR4 peptide antagonists include T140 and derivatives of T140. Exemplary derivatives of T140 include, but are not limited to, TN14003, TC14012, and TE14011 as well as those found in Tamamura, H. et al. Synthesis of potent CXCR4 inhibitors possessing low cytotoxicity and improved biostability based on T140 derivatives, Org. Biomol. Chem. 1:3656-3662, 2003, which is incorporated by reference herein in its entirety.

The term "therapeutically effective amount" as used herein refers to that amount of the compound being administered which will relieve to some extent one or more of the symptoms of the disorder being treated. In reference to cancer or pathologies related to unregulated cell division, a therapeutically effective amount refers to that amount which has the effect of (1) reducing the size of a tumor, (2) inhibiting (that is, slowing to some extent, preferably stopping) aberrant cell division, for example cancer cell division, (3) preventing or reducing the metastasis of cancer cells, and/or, (4) relieving to some extent (or, preferably, eliminating) one or more symptoms associated with a pathology related to or caused in part by unregulated or aberrant cellular division, including for example, cancer, or angiogenesis.

"Pharmaceutically acceptable salt" refers to those salts which retain the biological effectiveness and properties of the free bases and which are obtained by reaction with inorganic or organic acids such as hydrochloric acid, hydrobromic

acid, sulfuric acid, nitric acid, phosphoric acid, methanesulfonic acid, ethanesulfonic acid, p-toluenesulfonic acid, salicylic acid, malic acid, maleic acid, succinic acid, tartaric acid, citric acid, and the like.

A "pharmaceutical composition" refers to a mixture of one or more of the compounds described herein, or pharmaceutically acceptable salts thereof, with other chemical components, such as physiologically acceptable carriers and excipients. One purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

As used herein, a "pharmaceutically acceptable carrier" refers to a carrier or diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound.

An "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of a compound. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

"Treating" or "treatment" of a disease includes preventing the disease from occurring in an animal that may be predisposed to the disease but does not yet experience or exhibit symptoms of the disease (prophylactic treatment), inhibiting the disease (slowing or arresting its development), providing relief from the symptoms or side-effects of the disease (including palliative treatment), and relieving the disease (causing regression of the disease). With regard to cancer, these terms simply mean that the life expectancy of an individual affected with a cancer will be increased or that one or more of the symptoms of the disease will

be reduced.

The term "prodrug" refers to an agent, including nucleic acids and polypeptides, which is converted into a biologically active form *in vivo*. Prodrugs are often useful because, in some situations, they may be easier to administer than the parent compound. They may, for instance, be bioavailable by oral administration whereas the parent compound is not. The prodrug may also have improved solubility in pharmaceutical compositions over the parent drug. A prodrug may be converted into the parent drug by various mechanisms, including enzymatic processes and metabolic hydrolysis. Harper, N.J. (1962). Drug Latentiation in Jucker, ed. *Progress in Drug Research*, 4:221-294; Morozowich et al. (1977). Application of Physical Organic Principles to Prodrug Design in E. B. Roche ed. *Design of Biopharmaceutical Properties through Prodrugs and Analogs*, APhA; Acad. Pharm. Sci.; E. B. Roche, ed. (1977). *Bioreversible Carriers in Drug in Drug Design, Theory and Application*, APhA; H. Bundgaard, ed. (1985) *Design of Prodrugs*, Elsevier; Wang et al. (1999) Prodrug approaches to the improved delivery of peptide drug, *Curr. Pharm. Design*. 5(4):265-287; Pauletti et al. (1997). Improvement in peptide bioavailability: Peptidomimetics and Prodrug Strategies, *Adv. Drug. Delivery Rev.* 27:235-256; Mizen et al. (1998). The Use of Esters as Prodrugs for Oral Delivery of β-Lactam antibiotics, *Pharm. Biotech.* 11,:345-365; Gaignault et al. (1996). Designing Prodrugs and Bioprecursors I. Carrier Prodrugs, *Pract. Med. Chem.* 671-696; M. Asgharnejad (2000). Improving Oral Drug Transport Via Prodrugs, in G. L. Amidon, P. I. Lee and E. M. Topp, Eds., *Transport Processes in Pharmaceutical Systems*, Marcell Dekker, p. 185-218; Balant et al. (1990) Prodrugs for the improvement of drug

absorption via different routes of administration, *Eur. J. Drug Metab. Pharmacokinet.*, 15(2): 143-53; Balimane and Sinko (1999). Involvement of multiple transporters in the oral absorption of nucleoside analogues, *Adv. Drug Delivery Rev.*, 39(1-3):183-209; Browne (1997). Fosphenytoin (Cerebyx), *Clin. Neuropharmacol.* 20(1): 1-12; Bundgaard (1979). Bioreversible derivatization of drugs--principle and applicability to improve the therapeutic effects of drugs, *Arch. Pharm. Chemi.* 86(1): 1-39; H. Bundgaard, ed. (1985) *Design of Prodrugs,* New York: Elsevier; Fleisher et al. (1996). Improved oral drug delivery: solubility limitations overcome by the use of prodrugs, *Adv. Drug Delivery Rev.* 19(2): 115-130; Fleisher et al. (1985). Design of prodrugs for improved gastrointestinal absorption by intestinal enzyme targeting, *Methods Enzymol.* 112: 360-81; Farquhar D, et al. (1983). Biologically Reversible Phosphate-Protective Groups, *J. Pharm. Sci.*, 72(3): 324-325; Han, H.K. et al. (2000). Targeted prodrug design to optimize drug delivery, *AAPS PharmSci.*, 2(1): E6; Sadzuka Y. (2000). Effective prodrug liposome and conversion to active metabolite, *Curr. Drug Metab.*, 1(1):31-48; D.M. Lambert (2000) Rationale and applications of lipids as prodrug carriers, *Eur. J. Pharm. Sci.*, 11 Suppl 2:S15-27; Wang, W. et al. (1999) Prodrug approaches to the improved delivery of peptide drugs. *Curr. Pharm. Des.*, 5(4):265-87.

As used herein, the term "topically active agents" refers to compositions of the present disclosure that elicit pharmacological responses at the site of application (contact) to a host.

As used herein, the term "topically" refers to application of the compositions of the present disclosure to the surface of the skin and mucosal

cells and tissues.

The term "nucleic acid" is a term of art that refers to a string of at least two base-sugar-phosphate combinations. For naked DNA delivery, a polynucleotide contains more than 120 monomeric units since it must be distinguished from an oligonucleotide. However, for purposes of delivering RNA, RNAi and siRNA, either single or double stranded, a polynucleotide contains 2 or more monomeric units. Nucleotides are the monomeric units of nucleic acid polymers. The term includes deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) in the form of a messenger RNA, anti-sense, plasmid DNA, parts of a plasmid DNA or genetic material derived from a virus. Anti-sense is a polynucleotide that interferes with the function of DNA and/or RNA. Natural nucleic acids have a phosphate backbone, artificial nucleic acids may contain other types of backbones, but contain the same bases. RNA may be in the form of an tRNA (transfer RNA), snRNA (small nuclear RNA), rRNA (ribosomal RNA), mRNA (messenger RNA), anti-sense RNA, RNAi, siRNA, and ribozymes. The term also includes PNAs (peptide nucleic acids), phosphorothioates, and other variants of the phosphate backbone of native nucleic acids.

The term "siRNA" means a small inhibitory ribonucleic acid. The siRNA are typically less than 30 nucleotides in length and can be single or double stranded. The ribonucleotides can be natural or artificial and can be chemically modified. Longer siRNAs can comprise cleavage sites that can be enzymatically or chemically cleaved to produce siRNAs having lengths less than 30 nucleotides, typically 21 to 23 nucleotides. siRNAs share sequence homology with corresponding target mRNAs. The sequence homology can be 100 percent or

less but sufficient to result is sequence specific association between the siRNA and the targeted mRNA.

The term "inhibitory nucleic acid" means an RNA, DNA, or combination thereof that interferes or interrupts the translation of mRNA. Inhibitory nucleic acids can be single or double stranded. The nucleotides of the inhibitory nucleic acid can be chemically modified, natural or artificial.

The term "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result, such as modulation of CXCR4, SDF-1 activity. A prophylactically effective amount can be determined as described herein for an effective amount. Typically, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than a therapeutically effective amount.

The abbreviations used are: CXCR4, CXC Chemokine receptor-4; SDF-1, stromal-derived factor-1; FACS, fluorescence-activated cell sorter; VEGF, vascular endothelial growth factor; MTT, methylthiazoletetrazolium; RT-PCR, Reverse transcription Polymerase Chain Reaction; MAb, monoclonal antibody; PE, R-Phycoerithrin; SCID, Severe Combined Immunodeficient; CC_{50}, 50% cytotoxic concentration; EC_{50}, 50% effective concentration; SI, selective index (CC_{50}/EC_{50}); DCIS, Ductal carcinoma *in situ*, H&E, hematoxylin and eosin; siRNA, small interfering RNA; HPRT, hypoxanthine-guanine-phosphoribosyltransferase.

2. Exemplary Embodiments

Generally, the disclosure provides compositions and methods for treating or preventing a CXCR4 mediated pathology by administering a CXCR4 antagonist to a

host in a therapeutic amount, for example in an amount sufficient to inhibit CXCR4 signal transduction in a cell expressing a CXCR4 receptor or homologue thereof. Another embodiment provides uses of a CXCR4 antagonist for the manufacture of a medicament for the treatment of CXCR4 mediated pathologies including, but not limited to cancer. Still another embodiment provides uses of a CXCR4 peptide antagonist for the manufacture of medicament for the prevention of tumor cell metastasis in a mammal.

The CXCR4 antagonist compositions described here can be used to treat or prevent cancer, in particular the spread of cancer within an organism. Cancer is a general term for diseases in which abnormal cells divide without control. Cancer cells can invade nearby tissues and can spread through the bloodstream and lymphatic system to other parts of the body. It has been discovered that the administration of a CXCR4 antagonist to a host, for example a mammal, inhibits or reduces the metastasis of tumor cells, in particular breast cancer and prostate cancer.

There are several main types of cancer, and the disclosed compositions can be used to treat any type of cancer. For example, carcinoma is cancer that begins in the skin or in tissues that line or cover internal organs. Sarcoma is cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue. Leukemia is cancer that starts in blood-forming tissue such as the bone marrow, and causes large numbers of abnormal blood cells to be produced and enter the bloodstream. Lymphoma is cancer that begins in the cells of the immune system.

When normal cells lose their ability to behave as a specified, controlled and coordinated unit, a tumor is formed. A solid tumor is an abnormal mass of tissue that usually does not contain cysts or liquid areas. A single tumor may even have different

populations of cells within it with differing processes that have gone awry. Solid tumors may be benign (not cancerous), or malignant (cancerous). Different types of solid tumors are named for the type of cells that form them. Examples of solid tumors are sarcomas, carcinomas, and lymphomas. Leukemias (cancers of the blood) generally do not form solid tumors. The compositions described herein can be used to reduce, inhibit, or diminish the proliferation of tumor cells, and thereby assist in reducing the size of a tumor.

Representative cancers that may treated with the disclosed compositions and methods include, but are not limited to, bladder cancer, breast cancer, colorectal cancer, endometrial cancer, head & neck cancer, leukemia, lung cancer, lymphoma, melanoma, non-small-cell lung cancer, ovarian cancer, prostate cancer, testicular cancer, uterine cancer, cervical cancer, thyroid cancer, gastric cancer, brain stem glioma, cerebellar astrocytoma, cerebral astrocytoma, ependymoma, Ewing's sarcoma family of tumors, germ cell tumor, extracranial cancer, Hodgkin's disease, leukemia, acute lymphoblastic leukemia, acute myeloid leukemia, liver cancer, medulloblastoma, neuroblastoma, brain tumors generally, non-Hodgkin's lymphoma, osteosarcoma, malignant fibrous histiocytoma of bone, retinoblastoma, rhabdomyosarcoma, soft tissue sarcomas generally, supratentorial primitive neuroectodermal and pineal tumors, visual pathway and hypothalamic glioma, Wilms' tumor, acute lymphocytic leukemia, adult acute myeloid leukemia, adult non-Hodgkin's lymphoma, chronic lymphocytic leukemia, chronic myeloid leukemia, esophageal cancer, hairy cell leukemia, kidney cancer, multiple myeloma, oral cancer, pancreatic cancer, primary central nervous system lymphoma, skin cancer, small-cell lung cancer, among others.

A tumor can be classified as malignant or benign. In both cases, there is an

abnormal aggregation and proliferation of cells. In the case of a malignant tumor, these cells behave more aggressively, acquiring properties of increased invasiveness. Ultimately, the tumor cells may even gain the ability to break away from the microscopic environment in which they originated, spread to another area of the body (with a very different environment, not normally conducive to their growth) and continue their rapid growth and division in this new location. This is called metastasis. Once malignant cells have metastasized, achieving cure is more difficult. CXCR4 receptor antagonists are shown herein to modulate metastasis of cancer cells.

Benign tumors have less of a tendency to invade and are less likely to metastasize. They do divide in an uncontrolled manner, though. Depending on their location, they can be just as life threatening as malignant lesions. An example of this would be a benign tumor in the brain, which can grow and occupy space within the skull, leading to increased pressure on the brain. The compositions provided herein can be used to treat benign or malignant tumors.

2.1 CXCR4 Receptor and CXCR4 Receptor Antagonists

CXCR4 is a G-coupled heptahelical receptor which first drew attention as a major coreceptor for the entry of HIV. Activation of CXCR4 by SDF-1 results in activation of many downstream pathways including MAPK, PI3K, and calcium mobilization (Bleul, C. C. et al. The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. Nature, *382:* 829-833, 1996; Deng, H. K. Expression cloning of new receptors used by simian and human immunodeficiency viruses. Nature, 355: 296-300, 1997; Vlahakis, S. R. et al. G protein-coupled chemokine receptors induce both survival and apoptotic signaling pathways. J. Immunol., *169:* 5546-5554, 2002; Sotsios, Y. et al. The CXC chemokine stromal cell-

derived factor activates a Gi-coupled phosphoinositide 3-kinase in T lymphocytes. J. Immunol., *163:* 5954-5963, 1999; Kijowski, J. et al. The SDF-1-CXCR4 axis stimulates VEGF secretion and activates integrins but does not affect proliferation and survival in lymphohematopoietic cells. Stem Cells, *19:* 453-466. 2001; Rozmyslowicz, T. et al. T-lymphocytic cell lines for studying cell infectability by human immunodeficiency virus. Eur. J. Haematol., *67:* 142-151, 2001; Majka, M. Biological significance of chemokine receptor expression by normal human megakaryoblasts. Folia. Histochem. Cytobiol., *39:* 235-244, 2001; Majka, M. et al. Binding of stromal derived factor-1 alpha (SDF-1 alpha) to CXCR4 chemokine receptor in normal human megakaryoblasts but not in platelets induces phosphorylation of mitogen-activated protein kinase p42/44 (MAPK), ELK-1 transcription factor and serine/threonine kinase AKT. Eur. J. Haematol., *64:* 164-172, 2000). For hematopoietic stem cell activation, CXCR4 triggers migration to the marrow (Wright, D. E. et al. Hematopoietic stem cells are uniquely selective in their migratory response to chemokines. J. Exp. Med., *195:* 1145-1154, 2002; Voermans, C. et al. Migratory behavior of leukemic cells from acute myeloid leukemia patients. Leukemia, *16:* 650-657, 2002; Cashman, J. et al. Stromal-derived factor 1 inhibits the cycling of very primitive human hematopoietic cells in vitro and in NOD/SCID mice. Blood, *99:* 792-799, 2002; Spencer, A. et al. Enumeration of bone marrow homing haemopoietic stem cells from G-CSF- mobilised normal donors and influence on engraftment following allogeneic transplantation. Bone Marrow Transplant, *28:* 1019-1022, 2001; Vainchenker, W. Hematopoietic stem cells. Therapie, *56:* 379-381, 2001; Liesveld, J. L. et al. Response of human CD34+ cells to CXC, CC, and CX3C chemokines: implications for cell migration and activation. J. Hematother. Stem Cell

Res., *10:* 643-655, 2001; Lapidot, T. Mechanism of human stem cell migration and repopulation of NOD/SCID and B2mnull NOD/SCID mice. The role of SDF-1/CXCR4 interactions. Ann. N. Y. Acad. Sci., *938:* 83-95, 2001; Kollet, O. et al. T. Rapid and efficient homing of human CD34(+)CD38(-/low)CXCR4(+) stem and progenitor cells to the bone marrow and spleen of NOD/SCID andNOD/SCID/B2m(null) mice. Blood, *97:* 3283-3291, 2001) and directs peripheral blood cells into the lymph nodes and spleen (Blades, M. C. et al. Stromal cell-derived factor 1 (CXCL12) induces human cell migration into human lymph nodes transplanted into SCID mice. J. Immunol., *168:* 4308-4317, 2002). Together these results indicate that SDF-1/CXCR4, may play a "lock and key" function for directing cells to a variety of target organs. As CXCR4 is a major coreceptor for T-tropic HIV infection, a variety of compounds that target CXCR4 to prevent infection have been developed.

2.1.1 Peptide Antagonists

In various embodiments, the compounds recited in the disclosure are representative of the compounds that may be used therapeutically in formulations or medicaments for the treatment of CXCR4 mediated pathologies. One embodiment provides a method of treating a chemokine mediated pathology, or a pathology mediated by a receptor of the chemokine, in a mammal in need of such treatment, by administering to the mammal an effective amount of a chemokine receptor peptide antagonist, or a pharmaceutically acceptable salt or prodrug thereof. In another embodiment the chemokine is a chemokine that binds to the CXCR4 receptor. Exemplary chemokine mediated pathologies or pathologies mediated by a receptor of a chemokine include, but are not limited to, cancer. In a preferred embodiment, the chemokine receptor antagonist is a CXCR4 peptide antagonist such as

T140 or a derivative of T140 such as TN14003. The sequence of T140 is H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH (SEQ ID No.: 1) wherein Cit is L-citrulline, Nal is L-3-(2-naphthyl)alanine, and a disulfide bond links the two Cys residues. The sequence of TN14003 is H-Arg-Arg-Nal-Cys-Tyr-Cit-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-NH_2 (SEQ ID No.: 2), wherein Cit is L-citrulline, Nal is L-3-(2-naphthyl)alanine, and a disulfide bond links the two Cys residues. It will be appreciated that more than one peptide antagonist can be used in sequence or combination.

Representative CXCR4 peptide antagonists include but are not limited to TN14003, TC14012, TE14011, T140, T22, and derivatives, pharmaceutically acceptable salts, or prodrugs thereof as well as those found in Tamamura, H. et al. Synthesis of potent CXCR4 inhibitors possessing low cytotoxicity and improved biostability based on T140 derivatives, Org. Biomol. Chem. *1*:3656-3662, 2003, incorporated by reference in its entirety. CXCR4 peptide antagonists are known in the art. For example, Tamamura et al. (Tamamura, E. L. et al. Pharmacophore identification of a specific CXCR4 inhibitor, T140, leads to development of effective anti-HIV agents with very high selectivity indexes. Bioorg. Med. Chem. Lett., *10:* 2633-2637, 2000; Tamamura, H., et al. N. Conformational study of a highly specific CXCR4 inhibitor, T140, disclosing the close proximity of its intrinsic pharmacophores associated with strong anti-HIV activity. Bioorg. Med. Chem. Lett, *11:* 359-362. 2001) reported the identification of a specific CXCR4 inhibitor, T140, a 14-residue peptide that possessed a high level of anti-HIV activity and antagonism of T cell line-tropic HIV-1 entry among all antagonists of CXCR4 (Tamamura, E.L. et al. Pharmacophore identification of a specific CXCR4 inhibitor, T140, leads to development of effective anti-HIV agents with very high selectivity indexes. Bioorg. Med. Chem. Lett., *10:*

2633-2637, 2000; Tamamura, H. et al. Conformational study of a highly specific CXCR4 inhibitor, T140, disclosing the close proximity of its intrinsic pharmacophores associated with strong anti-HIV activity. Bioorg. Med. Chem. Lett, *11:* 359-362, 2001; Tamamura, H. et al. A low-molecular-weight inhibitor against the chemokine receptor CXCR4: a strong anti-HIV peptide T140. Biochem. Biophys. Res. Commun., *253:* 877-882, 1998) by mimicking SDF-1. Further improvements in the compound were achieved by amidating the C-terminal of T-140, and by reducing the total positive charges of the molecule by substituting basic residues with nonbasic polar amino acids. This resulted in the generation of a compound (TN14003) with properties which are far less cytotoxic and more stable in serum compared to T140 (Tamamura, H. Development of specific CXCR4 inhibitors possessing high selectivity indexes as well as complete stability in serum based on an anti-HIV peptide T140. Bioorg. Med. Chem. Lett, *11:* 1897-1902, 2001). The concentrations of T140 and TN14003 required for 50% protection of HIV-induced cytopathogenicity in MT-4 cells (EC_{50}) are 3.3 nM and 0.6 nM respectively. The concentrations of T140 and TN14003 that induce a 50% reduction of the viability of MT-4 cells (CC_{50}) are 59 μM and 410 μM respectively. These results reflect the improved therapeutic index for TN14003 over T140 ($SI_{TN14003}$=680,000; SI_{T140}=17,879; $SI=CC_{50}/EC_{50}$). The sequence of T22 is RRWCYRKCYKGYCYRKCR (SEQ ID NO: 3).

Still another embodiment provides a method of treating cancer by administering to a host, such as a mammal, in need of such treatment a tumor inhibiting amount of CXCR4 antagonist, for example a peptide CXCR4 antagonist, a pharmaceutically acceptable salt or prodrug thereof.

Yet another embodiment provides a method for preventing tumor cell

metastasis in a mammal by administering a metastasis inhibiting amount of a CXCR4 antagonist, for example a peptide antagonist, pharmaceutically acceptable salt or prodrug thereof. Inhibiting metastasis means preventing or reducing the spread of cancer from a tumor origination site to a secondary site in an organism.

Still another embodiment provides a method for treating or preventing metastasis of a non-hematopoietic cancer or tumor by administering an anti-metastasis amount of a CXCR4 antagonist, for example a peptide antagonist, to a host such as a mammal in need of such treatment.

In one embodiment, the CXCR4 antagonist is TN14003 which binds to the SDF-1 binding site of CXCR4 protein. As provided herein, fluorescence staining of CXCR4 using biotin-labeled CXCR4 antagonist on cells pretreated with SDF-1α for 10 min followed by cold-acetone fixation show that TN14003 binds the SDF-1 binding site of CXCR4. The pretreatment of cells with SDF-1α may induce endocytosis of CXCR4 receptors. Because cells were only treated with SDF-1α for short time, some CXCR4 proteins should be in a process of endocytosis and the others still remained on cell surface. The immunofluorescence of the biotin-labeled CXCR4 antagonist was negative in both membrane and cytosol in the cells pretreated with SDF-1α for 10 min (Figure 1A right). Therefore, the data provided herein demonstrates that CXCR4 antagonist binds to the SDF-1 binding site of CXCR4 protein.

In vitro invasion assays showed that CXCR4-negative MDA-MB-435 cells could not invade through matrigel while CXCR4-positive MDA-MB-231 cells did in the presence of SDF-lα at the bottom chamber (Figure 2). Furthermore, MDA-MB-435 cells could not invade through matrigel even in the presence of 1% FBS at the bottom chamber while MDA-MB-231 cells did (data not shown). These results

indicate that CXCR4 expression is required for *in vitro* matrigel invasion. To increase the probability to form metastasis in the animal model, 2×10^6 tumor cells were intravenously administered twice (day 0 and day 6) to female SCID mice supplemented with 17β-estradiol (60-day release pill). All animals treated with the control peptide twice weekly for 55 days developed lung metastasis. On the other hand, CXCR4 antagonist treated animals failed to form visible lung metastasis. Semi-quantitative Real-Time RT-PCR revealed that four out of seven in the CXCR4 antagonist treated group contained some micrometastasis in their lungs (Figure 3). This decreased metastasis to lung in CXCR4 antagonist treated animals was not due to a cytotoxicity of antagonist because the CXCR4 antagonist did not affect cell proliferation even at 10 nM concentration (Figure 4). In addition, H & E staining of liver and kidney tissues from mice treated with CXCR4 antagonist did not exhibit any central necrosis in the liver or tubular necrosis in the kidney. The CXCR4 antagonist was evaluated for toxicity on hemopoietic progenitor cell colony formation and, at 10 nM, the highest concentration tested, there was no discernable effect on hemopoietic progenitor cell colony formation. Therefore, CXCR4 antagonists described herein can be used as an excellent therapeutic agent to inhibit breast cancer metastasis.

The data provided herein demonstrates that CXCR4/SDF-1 interaction is one of the major requirements for breast cancer metastasis. The elevated level of CXCR4 in primary tumors correlates with the metastatic potential of tumors. CXCR4 overexpression has been found in other tumors besides breast cancer, such as brain tumors (Rempel, S. A. et al. Identification and localization of the cytokine SDF1 and its receptor, CXC chemokine receptor 4, to regions of necrosis and angiogenesis in human glioblastoma. Clin. Cancer Res., *6:* 102-111, 2000; Sehgal,

A. et al. CXCR-4, a chemokine receptor, is overexpressed in and required for proliferation of glioblastoma tumor cells. J. Surg. Oncol., *69:* 99-104, 1998; Sehgal, A. et al. Molecular characterization of CXCR-4: a potential brain tumor-associated gene. J. Surg. Oncol., *69:* 239-248, 1998), pancreatic cancer (Koshiba, T. et al. Expression of stromal cell-derived factor 1 and CXCR4 ligand receptor system in pancreatic cancer: a possible role for tumor progression. Clin. Cancer Res., *6:* 3530-3535, 2000), ovarian epithelial tumors (Scotton, C. J. et al. Epithelial cancer cell migration: a role for chemokine receptors? Cancer Res., *61:* 4961-4965, 2001), prostate cancer (Taichman, R. S. Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. Cancer Res., *62:* 1832-1837, 2002), kidney cancer (Schrader, A. J. et al. CXCR4/CXCL12 expression and signalling in kidney cancer. Br. J. Cancer, *86:* 1250-1256, 2002), and non-small cell lung cancer (Takanami, I. Overexpression of CCR7 mRNA in nonsmall cell lung cancer: correlation with lymph node metastasis. Int. J. Cancer, *105:* 186-189, 2003).

Accordingly, embodiments of the present disclosure include methods of treating breast, brain, pancreatic, ovarian, prostate, kidney, and non-small lunch cancer. In particular, metastasis of breast, brain, pancreatic, ovarian, prostate, kidney, and non-small lunch cancer can be reduced or inhibited by administering a CXCR4 peptide antagonist, such as TN14003, to host in need of such treatment in an anti-metastasis effective amount.

Neutralizing CXCR4/SDF-1 activation with the CXCR4 antibody impaired breast cancer metastasis to the lymph node and lung in animal models for breast cancer metastasis (Muller, A. Involvement of chemokine receptors in breast cancer metastasis. Nature, *410:* 50-56, 2001), and similar results have been observed in

prostate cancer bone metastasis. As shown herein, a synthetic 14-mer peptide blocked the CXCR4 receptor binding to its ligand SDF-1 and inhibited CXCR4/SDF-1 mediated invasion in *vitro* and metastasis *in vivo* with a higher specificity than anti-CXCR4 antibodies (R & D Systems). The anti-invasion and anti-metastasis activity of this peptide correlated well with their inhibitory activity on SDF-1α binding to CXCR4. This antagonist is proven safe by proliferation assay, animal histology, and hemopoietic progenitor cell colony formation. Thus, the CXCR4 antagonist TN14003 is an effective therapeutic agent of breast cancer metastasis as well as inhibitors of T-tropic HIV infection.

Anti-CXCR4 antibody is capable of decreasing breast cancer metastasis at high concentrations *in vivo* (50 mg/kg) (Muller, A. Involvement of chemokine receptors in breast cancer metastasis. Nature, *410:* 50-56, 2001). However, antibody therapy may be limited by: (i) the difficulty or expense of commercial-scale production; (ii) delivery problem and slow diffusion due to a large mass; and (ii) exclusion of monoclonal antibody from compartments like the blood/brain barrier (Cho, M. J. and Juliano, R. Macromolecular versus small-molecule therapeutics: drug discovery, development and clinical considerations. Trends Biotechnol, *14:* 153-158, 1996). For example it has been theorized that in a compact tumor mass, large molecules such as antibodies with a molecular weight of 150kDa may not easily diffuse between cells inside of the solid tumor. Therefore, antibodies may be inefficient molecules to target cells deep within the tumor mass. Especially, the use of antibody (150 kDa) or antibody fragments ($F(ab')_2$, 30 kDa) as an imaging probe for Positron Emission Tomography (PET) or Single Photon Emission Computed Tomography (SPECT) to detect CXCR4 positive tumors is not practical. This is

because PET or SPECT nuclides have short half lives (20-109 minutes) while antibody or antibody fragments will take a long time (at least 24 hours) to reach the target site (tumor) and clear out of the blood and tissues.

2.1.2 Small Molecule Antagonists

In other embodiments, the CXCR4 antagonist is a non-peptide compound. Representative small molecule antagonists include but are not limited to KRH-1636, N-{(S)-4-guanidino-1-[(S)-1-naphthalen-1-yl-ethylcarbamoyl]butyl}-4-{[(pyridin-2-yl-methyl)amino]methyl}benzamide (Ichiyama, K. et al. A duodenally absorbable CXC chemokine receptor 4 antagonist, KRH-1636, exhibits a potent and selective anti-HIV-1 activity, 2003, PNAS 100(7): 4185-4190).

AMD3100 also known as 1,1'-[1,4-phenylenebis(methylene)]-bis-1,4, 8,11-tetraazacyclotetradecane octahydrochloride dihydrate is a non-peptide CXCR4 receptor antagonist and is a potent blocker of human immunodeficiency virus cell entry. AMD3100 is a symmetrical bicyclam composed of two identical 1,4,8,11-tetraazacyclotetradecane (cyclam) moieties connected by a relatively rigid phenylenebismethylene linker (Gerlach, L.O. et al. Molecular interactions of cyclam and bicyclam non-peptide antagonists with the CXCR4 chemokine receptor. J Biol Chem. 2001 Apr 27;276(17):14153-60). Another CXCR4 antagonist includes T134 (Arakaki, R. T134, a small-molecule CXCR4 inhibitor, has no cross-drug resistance with AMD3100, a CXCR4 antagonist with a different structure. Journal of Virology, February 1999, p. 1719-1723, Vol. 73, No. 2).

2.2 Polynucleotide Antagonists

Another embodiment provides a polynucleotide CXCR4 antagonist that inhibits, reduces, or prevents the expression of CXCR4 polypeptides in a cell. In particular,

siRNA polynucleotides directed to CXCR4 have been discovered to prevent the metastasis of cancer in a host organism, for example a mammal.

Another embodiment provides a method for treating a chemokine-related or chemokine receptor-related pathology, such as cancer, by administering to a host in need of such treatment, a therapeutic amount of one or more siRNAs specific for CXCR4 polynucleotides such as CXCR4 mRNA or a fragment thereof.

The inhibitory nucleic acids of certain embodiments of the present disclosure are directed to inhibiting or interfering with the expression of proteins involved in the CRCX4 signal transduction pathway. The inhibitory nucleic acids disclosed herein include small inhibitory ribonucleic acids (siRNAs) that are typically less than 30 nucleotides in length, more typically 19-21 or 19-23 nucleotides in length, and can be single or double stranded. One strand of a double-stranded siRNA comprises at least a partial sequence complementary to a target mRNA, for example CXCR4 mRNA. The ribonucleotides of the siRNA can be natural or artificial and can be chemically modified, for example to resist enzymatic degradation or modulate solubility or bioavailability. Longer siRNAs can comprise cleavage sites that can be enzymatically or chemically cleaved to produce siRNAs having lengths less than 30 nucleotides. The phosphate backbones of the siRNAs can be chemically modified to resist enzymatic degradation. The sequence homology can be about 100 percent or less, typically from about 100-90 percent, but sufficient to result is sequence specific association between the siRNA and the targeted mRNA.

Cancer cells acquire CXCR4 overexpression before they leave the primary site and migrate toward organs with high SDF-1 levels. It has been discovered that blocking CXCR4 expression, for example at the mRNA level using small interfering RNAs

(siRNAs) impaired invasion of breast cancer cells in matrigel invasion assay and inhibited breast cancer metastasis in an animal model. Moreover, it has been discovered that direct injection of a pool of naked siRNA duplexes can prevent tumorigenesis in vivo.

RNA interference is a cellular mechanism in which double-stranded RNA triggers the silencing of the corresponding cellular gene (Fire, A. et al. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806-11, 1998; Sharp, P.A. RNA interference-2001. *Genes Dev* 15, 485-90, 2001; Plasterk, R.H. RNA silencing: the genome's immune system. *Science* 296, 1263-5, 2002). The double strand RNA (dsRNA) in the cell is processed into short, approximately 21-22 nucleotide dsRNAs termed small interfering RNAs (siRNA) (Elbashir, S.M. et al. Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. *Embo* J20, 6877-88, 2001; Hamilton, A.J. & Baulcombe, B.C. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* 286, 950-2, 1999). A major breakthrough in the application of RNA interference technology in mammalian cells came from the development of a 21-22 nucleotide synthetic siRNAs to silence targeted genes in mammalian cells (Elbashir, S.M., Harborth, J., Weber, K. & Tuschl, T. Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods* 26, 199-213, 2002; Elbashir, S.M. et al. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411,** 494-8, 2001). Recently, it has been shown that duplex siRNA can be effectively delivered to into the target cells without any kind of carriers by the tail vein injection (Lewis, D. L. et al. Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. *Nat*

Genet 32, 107-8, 2002; Song, E. et al. RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat Med* 9, 347-51, 2003). Sorensen et al. also showed that cationic liposome-based intravenous injection in mice of plasmid encoding the green fluorescent protein (GFP) with its cognate siRNA, inhibited GFP gene expression in various organs (Sorensen, D. R. et al. Gene silencing by systemic delivery of synthetic siRNAs in adult mice. J Mol Biol 327, 761-6, 2003).

Current models of RNA interference divide the process of inhibition into broad "initiation" and "effector" stages. In the initiation step, input dsRNA is digested into 21-23 nucleotide small interfering RNAs (siRNAs), which have also been called "guide RNAs." Evidence indicates that siRNAs are produced when the enzyme Dicer, a member of the RNase III family of dsRNA-specific ribonucleases, processively cleaves dsRNA in an ATP-dependent, processive manner. Successive cleavage events degrade the RNA to 19-21 bp duplexes (siRNAs), each with 2-nucleotide 3' overhangs. Inhibitory nucleic acids of the present disclosure can be enzymatically cleaved, for example in vivo, to produce siRNAs from 10 to about 30 nucleotides, typically about 19 to about 23 nucleotides.

In the effector step, the siRNA duplexes bind to a nuclease complex to form what is known as the RNA-induced silencing complex, or RISC. An ATP-depending unwinding of the siRNA duplex is required for activation of the RISC. The active RISC then targets the homologous transcript by base pairing interactions and cleaves the mRNA ~12 nucleotides from the 3' terminus of the siRNA. Although the mechanism of cleavage is at this date unclear, research indicates that each RISC contains a single siRNA and an RNase that appears to be distinct from Dicer. Because of the remarkable potency of RNAi in some organisms, an amplification step within the RNAi pathway has

also been proposed. Amplification could occur by copying of the input dsRNAs, which would generate more siRNAs, or by replication of the siRNAs themselves. Alternatively or in addition, amplification could be effected by multiple turnover events of the RISC. One embodiment encompasses the in vivo amplification of the siRNAs disclosed herein. Additionally, the siRNAs described herein can form a complex with additional proteins and/or cofactors to enzymatically cleave a target mRNA.

Another embodiment provides a method for treating cancer by administering to a host in need thereof, a metastasis-inhibiting amount of pharmaceutical composition comprising one or more, typically at least two, siRNAs specific to CXCR4. It has been discovered that using one or a combination of siRNAs specific to CXCR4 effectively suppresses CXCR4 expression to prevent tumorigenesis. In a preferred embodiment, the siRNAs are specific for and/or contain the following cDNA sequence segments of CXCR4: ^{197}AATAAAATCTTCCTGCCCACC^{217} (SEQ ID NO: 4) and/or ^{529}AAGGAAGCTGTTGGCTGAAAA^{549} (SEQ ID NO: 5) or complementary or antisense sequences thereof. It will be appreciated that RNA sequences replace T for U. In some embodiments, the disclosed siRNAs duplexes include 3' overhanging nucleotides, for example uridine dimers.

Other CXCR4 cDNA target sequences for siRNA duplex generation include, but are not limited to: TAACTACACCGAGGAAATG (SEQ ID NO: 6); TCTTCTTAACTGGCATTGT (SEQ ID NO: 7); TCTTTGCCAACGTCAGTGA (SEQ ID NO: 8); GTTTCAGCACATCATGGTT (SEQ ID NO: 9); CATCATGGTTGGCCTTATC (SEQ ID NO: 10); TCCTGCCTGGTATTGTCAT (SEQ ID NO: 11); TCCTGTCCTGCTATTGCAT (SEQ ID NO: 12);

GCATCGACTCCTTCATCCT (SEQ ID NO: 13); GGAAAGCGAGGTGGACATT (SEQ ID NO: 14) or complementary or antisense sequences thereof.

Another embodiment provides a method for treating a chemokine related or chemokine receptor related pathology, for example cancer, including administering to a host in need of such treatment a pharmaceutical composition comprising an siRNA containing the following sequence ^{197}UAAAAUCUUCCUGCCCACCdTdT^{217} (SEQ ID NO: 15) or ^{529}GGAAGCUGUUGGCUGAAAAdTdT^{549} (SEQ ID NO: 16), a combination thereof, pharmaceutically acceptable salts, or prodrugs thereof, in an amount sufficient to inhibit translation of CXCR4 mRNA. In some embodiments, the siRNA compositions are delivered "naked" i.e., without a vector or packaging vehicle such as proteins, lipids, expression vectors or liposomes.

Still another embodiment provides a siRNA composition linked to a targeting moiety. Targeting moieties include, but are not limited to, substances that facilitate the delivery of the siRNA to a specific target. Representative targeting moieties include, but are not limited to, folate, derivatives of folate or folate receptors, polysaccharides such as pullulan, sugars such as galactose, antibodies specific for surface proteins or polypeptides, or small molecule ligands of cell surface receptors.

2.3 Combination Therapy

The disclosed compositions can be used to treat a pathology, for example a proliferative pathology such as cancer or other chemokine related pathology independently or in combination with one another or with one or more additional therapeutic agents. Representative therapeutic agents include but are not limited

to antibiotics, anti-inflammatories, anti-oxidants, analgesics, radioisotopes, chemotherapeutic agents such as nascopine, paclitaxel, nocodazole, vinca alkaloids, adriamycin, alkeran, Ara-C, BiCNU, busulfan, CCNU, carboplatinum, cisplatinum, cytoxan, daunorubicin, DTIC, 5-FU, fludarabine, hydrea, idarubicin, ifosfamide, methotrexate, mithramycin, mitomycin, mitoxantrone, nitrogen, mustard, velban, vincristine, VP-16, gemcitabine (gemzar), herceptin, irinotecan, (camptosar, CPT-11), leustatin, navelbine, rituxan, STI-571, taxotere, topotecan, (hycamtin), xeloda (capecitabine), zevelin, and combinations thereof.

It will be appreciated that the compounds of the present disclosure can be used in combination with radiation therapy or surgical procedures for the treatment of a pathology, for example cancer.

2.4 Diagnostics

Another embodiment provides compositions and methods for the detection and diagnosis of chemokine or chemokine receptor mediated pathologies, including, but not limited to, cancer and cancer metastasis. For example, a CXCR4 peptide antagonist, for example TN14003, can be labeled with a detectable label. The detectable label can be a radioactive isotope, substrate producing enzyme or substrate for an enzyme, metal, bead, metal particle, fluorophore, phosphor, biotin, dyes, or other moiety that can produce a detectable signal or can be detected using known techniques. An exemplary label is fluorine-18. One or more detectable labels can be used for example to generate a fluorescence resonance energy transfer system (FRET). Chemical modification of peptides is known in the art (see for example, www.probes.com). Exemplary fluorophores that can be used are found in the Molecular Probes Catalogue which is incorporated by reference herein in its entirety.

Another embodiment provides a method for detecting a cancer cell or cancer cell metastasis including contacting a cell sample with a CXCR4 antagonist comprising a detectable label, for example a CXCR4 peptide antagonist including, but not limited to TN14003, detecting the detectable label, and correlating the amount of detectable label with the presence of cancer cells or cancer cell metastasis.

Another embodiment provides a method for detecting a cancer cell or cancer cell metastasis including contacting a cell sample with a fluorescently labeled CXCR4 peptide antagonist such as TN14003, irradiating the cell sample comprising the fluorescently labeled CXCR4 peptide antagonist with an exciting amount of electromagnetic radiation, detecting the emission of the fluorescently labeled CXCR4, and correlating the detectable fluorescence with the presence of cancer cells or cancer cell metastasis.

Yet another embodiment provides a method for detecting a cancer cell or cancer cell metastasis including contacting a cell sample with a fluorescently labeled CXCR4 peptide antagonist such as TN14003, irradiating the cell sample comprising the fluorescently labeled CXCR4 peptide antagonist with an exciting amount of electromagnetic radiation, detecting the emission of the fluorescently labeled CXCR4, and correlating the detectable fluorescence with the presence of cancer cells or cancer cell metastasis.

It will be appreciated that the cell sample in the various embodiments can also be contacted with a reagent for detecting a second determinant indicative of cancer or metastasis. An exemplary second determinant indicative of metastasis includes, but is not limited to the Her-2 protein or fragment thereof. Accordingly, an antibody that is specific for Her-2 can also be used in the disclosed methods for detecting cancer or

cancer cell metastasis. Other markers indicative of cancer or metastasis include, but are not limited to, *BCR-abl, ALL1, AML1, CBF-β* gene, *PML-RARA, p53, CD20*, IL-2 receptor-α, thymidylate synthase, estrogen receptor, progesterone receptor, androgen receptor, *ras, PGY1, EGFR, VEGF*, platelet-derived growth factor receptor, JAK kinases, fibroblast growth factor receptor, and phosphatyidylinositol-3' kinase.

Still another embodiment provides a method for detecting a cancer cell or cancer cell metastasis including contacting a cell sample with a CXCR4 antagonist, for example a peptide antagonist such as TN14003, comprising a first label, contacting the CXCR4 antagonist having a first label with a second label, detecting the second label, and correlating the amount of the second label with the presence of cancer cells or cancer cell metastasis. The first label can be biotin, and the second label can be streptavidin conjugated with a detectable label such as a fluorophore. It has been found that biotinylated CXCR4 antagonists can be useful as a quantitative diagnostic tool to identify CXCR4 receptor positive tumors in culture and clinical samples (Figure 1).

It has been discovered that biotinylated CXCR4 antagonist is a potent reagent for detecting CXCR4 receptors from cultured cancer cells and paraffinized tissues on breast cancer patients through the use of immunofluorescence and flow cytometry. The combined usage of the CXCR4 antagonist and other antibodies of known metastatic markers (e.g., Her-2) can be used for the detection of cancer and cancer metastasis, including but not limited to breast cancer metastasis.

2.5. Pharmaceutical Compositions

Pharmaceutical compositions and dosage forms of the disclosure comprise a pharmaceutically acceptable salt of compounds of an antagonist of CXCR4 or a pharmaceutically acceptable polymorph, solvate, hydrate, dehydrate, co-crystal,

anhydrous, or amorphous form thereof. Specific salts of an antagonist of CXCR4 include, but are not limited to, sodium, lithium, potassium salts, and hydrates thereof.

Pharmaceutical compositions and unit dosage forms of the disclosure typically also comprise one or more pharmaceutically acceptable excipients or diluents. Advantages provided by specific compounds of the disclosure, such as, but not limited to, increased solubility and/or enhanced flow, purity, or stability (e.g., hygroscopicity) characteristics can make them better suited for pharmaceutical formulation and/or administration to patients than the prior art.

Pharmaceutical unit dosage forms of the compounds of this disclosure are suitable for oral, mucosal (e.g., nasal, sublingual, vaginal, buccal, or rectal), parenteral (e.g., intramuscular, subcutaneous, intravenous, intraarterial, or bolus injection), topical, or transdermal administration to a patient. Examples of dosage forms include, but are not limited to: tablets; caplets; capsules, such as hard gelatin capsules and soft elastic gelatin capsules; cachets; troches; lozenges; dispersions; suppositories; ointments; cataplasms (poultices); pastes; powders; dressings; creams; plasters; solutions; patches; aerosols (e.g., nasal sprays or inhalers); gels; liquid dosage forms suitable for oral or mucosal administration to a patient, including suspensions (e.g., aqueous or non-aqueous liquid suspensions, oil-in-water emulsions, or water-in-oil liquid emulsions), solutions, and elixirs; liquid dosage forms suitable for parenteral administration to a patient; and sterile solids (e.g., crystalline or amorphous solids) that can be reconstituted to provide liquid dosage forms suitable for parenteral administration to a patient.

The composition, shape, and type of dosage forms of the compositions of

the disclosure will typically vary depending on their use. For example, a dosage form used in the acute treatment of a disease or disorder may contain larger amounts of the active ingredient, for example a CXCR4 antagonist or combinations thereof, than a dosage form used in the chronic treatment of the same disease or disorder. Similarly, a parenteral dosage form may contain smaller amounts of the active ingredient than an oral dosage form used to treat the same disease or disorder. These and other ways in which specific dosage forms encompassed by this disclosure will vary from one another will be readily apparent to those skilled in the art. See, e.g., Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing, Easton, Pa. (1990).

Typical pharmaceutical compositions and dosage forms comprise one or more excipients. Suitable excipients are well known to those skilled in the art of pharmacy or pharmaceutics, and non-limiting examples of suitable excipients are provided herein. Whether a particular excipient is suitable for incorporation into a pharmaceutical composition or dosage form depends on a variety of factors well known in the art including, but not limited to, the way in which the dosage form will be administered to a patient. For example, oral dosage forms such as tablets or capsules may contain excipients not suited for use in parenteral dosage forms. The suitability of a particular excipient may also depend on the specific active ingredients in the dosage form. For example, the decomposition of some active ingredients can be accelerated by some excipients such as lactose, or when exposed to water. Active ingredients that comprise primary or secondary amines are particularly susceptible to such accelerated decomposition.

The disclosure further encompasses pharmaceutical compositions and

dosage forms that comprise one or more compounds that reduce the rate by which an active ingredient will decompose. Such compounds, which are referred to herein as "stabilizers," include, but are not limited to, antioxidants such as ascorbic acid, pH buffers, or salt buffers. In addition, pharmaceutical compositions or dosage forms of the disclosure may contain one or more solubility modulators, such as sodium chloride, sodium sulfate, sodium or potassium phosphate or organic acids. A specific solubility modulator is tartaric acid.

Like the amounts and types of excipients, the amounts and specific type of active ingredient in a dosage form may differ depending on factors such as, but not limited to, the route by which it is to be administered to patients. However, typical dosage forms of the compounds of the disclosure comprise a pharmaceutically acceptable salt of an antagonist of CXCR4, or a pharmaceutically acceptable polymorph, solvate, hydrate, dehydrate, co-crystal, anhydrous, or amorphous form thereof, in an amount of from about 10 mg to about 1000 mg, preferably in an amount of from about 25 mg to about 750 mg, and more preferably in an amount of from 50 mg to 500 mg.

2.5.1. Oral Dosage Forms

Pharmaceutical compositions of the disclosure that are suitable for oral administration can be presented as discrete dosage forms, such as, but not limited to, tablets (including without limitation scored or coated tablets), pills, caplets, capsules, chewable tablets, powder packets, cachets, troches, wafers, aerosol sprays, or liquids, such as but not limited to, syrups, elixirs, solutions or suspensions in an aqueous liquid, a non-aqueous liquid, an oil-in-water emulsion, or a water-in-oil emulsion. Such compositions contain a predetermined amount of

the pharmaceutically acceptable salt of a CXCR4 antagonist, and may be prepared by methods of pharmacy well known to those skilled in the art. See generally, Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing, Easton, Pa. (1990).

Typical oral dosage forms of the compositions of the disclosure are prepared by combining the pharmaceutically acceptable salt of a CXCR4 antagonist in an intimate admixture with at least one excipient according to conventional pharmaceutical compounding techniques. Excipients can take a wide variety of forms depending on the form of the composition desired for administration. For example, excipients suitable for use in oral liquid or aerosol dosage forms include, but are not limited to, water, glycols, oils, alcohols, flavoring agents, preservatives, and coloring agents. Examples of excipients suitable for use in solid oral dosage forms (e.g., powders, tablets, capsules, and caplets) include, but are not limited to, starches, sugars, microcrystalline cellulose, kaolin, diluents, granulating agents, lubricants, binders, and disintegrating agents.

Due to their ease of administration, tablets and capsules represent the most advantageous solid oral dosage unit forms, in which case solid pharmaceutical excipients are used. If desired, tablets can be coated by standard aqueous or nonaqueous techniques. These dosage forms can be prepared by any of the methods of pharmacy. In general, pharmaceutical compositions and dosage forms are prepared by uniformly and intimately admixing the active ingredient(s) with liquid carriers, finely divided solid carriers, or both, and then shaping the product into the desired presentation if necessary.

For example, a tablet can be prepared by compression or molding. Compressed tablets can be prepared by compressing in a suitable machine the active ingredient(s) in a free-flowing form, such as a powder or granules, optionally mixed with one or more excipients. Molded tablets can be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent.

Examples of excipients that can be used in oral dosage forms of the disclosure include, but are not limited to, binders, fillers, disintegrants, and lubricants. Binders suitable for use in pharmaceutical compositions and dosage forms include, but are not limited to, corn starch, potato starch, or other starches, gelatin, natural and synthetic gums such as acacia, sodium alginate, alginic acid, other alginates, powdered tragacanth, guar gum, cellulose and its derivatives (e.g., ethyl cellulose, cellulose acetate, carboxymethyl cellulose calcium, sodium carboxymethyl cellulose), polyvinyl pyrrolidone, methyl cellulose, pre-gelatinized starch, hydroxypropyl methyl cellulose, (e.g., Nos. 2208, 2906, 2910), microcrystalline cellulose, and mixtures thereof.

Suitable forms of microcrystalline cellulose include, but are not limited to, the materials sold as AVICEL-PH-101, AVICEL-PH-103 AVICEL RC-581, and AVICEL-PH-105 (available from FMC Corporation, American Viscose Division, Avicel Sales, Marcus Hook, Pa., U.S.A.), and mixtures thereof. An exemplary suitable binder is a mixture of microcrystalline cellulose and sodium carboxymethyl cellulose sold as AVICEL RC-581. Suitable anhydrous or low moisture excipients or additives include AVICEL-PH-103™ and Starch 1500 LM.

Examples of fillers suitable for use in the pharmaceutical compositions and

dosage forms disclosed herein include, but are not limited to, talc, calcium carbonate (e.g., granules or powder), microcrystalline cellulose, powdered cellulose, dextrates, kaolin, mannitol, silicic acid, sorbitol, starch, pre-gelatinized starch, and mixtures thereof. The binder or filler in pharmaceutical compositions of the disclosure is typically present in from about 50 to about 99 weight percent of the pharmaceutical composition or dosage form.

Disintegrants are used in the compositions of the disclosure to provide tablets that disintegrate when exposed to an aqueous environment. Tablets that contain too much disintegrant may swell, crack, or disintegrate in storage, while those that contain too little may be insufficient for disintegration to occur and may thus alter the rate and extent of release of the active ingredient(s) from the dosage form. Thus, a sufficient amount of disintegrant that is neither too little nor too much to detrimentally alter the release of the active ingredient(s) should be used to form solid oral dosage forms of the disclosure. The amount of disintegrant used varies based upon the type of formulation and mode of administration, and is readily discernible to those of ordinary skill in the art. Typical pharmaceutical compositions comprise from about 0.5 to about 15 weight percent of disintegrant, preferably from about 1 to about 5 weight percent of disintegrant.

Disintegrants that can be used to form pharmaceutical compositions and dosage forms of the disclosure include, but are not limited to, agar--agar, alginic acid, calcium carbonate, microcrystalline cellulose, croscarmellose sodium, crospovidone, polacrilin potassium, sodium starch glycolate, potato or tapioca starch, other starches, pre-gelatinized starch, clays, other algins, other celluloses, gums, and mixtures thereof.

Lubricants that can be used to form pharmaceutical compositions and dosage forms of the disclosure include, but are not limited to, calcium stearate, magnesium stearate, mineral oil, light mineral oil, glycerin, sorbitol, mannitol, polyethylene glycol, other glycols, stearic acid, sodium lauryl sulfate, talc, hydrogenated vegetable oil (e.g., peanut oil, cottonseed oil, sunflower oil, sesame oil, olive oil, corn oil, and soybean oil), zinc stearate, ethyl oleate, ethyl laureate, agar, and mixtures thereof. Additional lubricants include, for example, a syloid silica gel (AEROSIL 200, manufactured by W. R. Grace Co. of Baltimore, Md.), a coagulated aerosol of synthetic silica (marketed by Degussa Co. of Plano, Tex.), CAB-O-SIL (a pyrogenic silicon dioxide product sold by Cabot Co. of Boston, Mass.), and mixtures thereof. If used at all, lubricants are typically used in an amount of less than about 1 weight percent of the pharmaceutical compositions or dosage forms into which they are incorporated.

This disclosure further encompasses lactose-free pharmaceutical compositions and dosage forms, wherein such compositions preferably contain little, if any, lactose or other mono- or di-saccharides. As used herein, the term "lactose-free" means that the amount of lactose present, if any, is insufficient to substantially increase the degradation rate of an active ingredient.

Lactose-free compositions of the disclosure can comprise excipients which are well known in the art and are listed in the USP (XXI)/NF (XVI), which is incorporated herein by reference. In general, lactose-free compositions comprise a pharmaceutically acceptable salt of a CXCR4 antagonist, a binder/filler, and a lubricant in pharmaceutically compatible and pharmaceutically acceptable amounts. Preferred lactose-free dosage forms comprise a pharmaceutically

acceptable salt of a CXCR4 antagonist, microcrystalline cellulose, pre-gelatinized starch, and magnesium stearate.

This disclosure further encompasses anhydrous pharmaceutical compositions and dosage forms comprising active ingredients, since water can facilitate the degradation of some compounds. For example, the addition of water (e.g., 5%) is widely accepted in the pharmaceutical arts as a means of simulating long-term storage in order to determine characteristics such as shelf life or the stability of formulations over time. See, e.g., Jens T. Carstensen, Drug Stability: Principles & Practice, 379-80 (2nd ed., Marcel Dekker, NY, N.Y.: 1995). Water and heat accelerate the decomposition of some compounds. Thus, the effect of water on a formulation can be of great significance since moisture and/or humidity are commonly encountered during manufacture, handling, packaging, storage, shipment, and use of formulations.

Anhydrous pharmaceutical compositions and dosage forms of the disclosure can be prepared using anhydrous or low moisture containing ingredients and low moisture or low humidity conditions. Pharmaceutical compositions and dosage forms that comprise lactose and at least one active ingredient that comprises a primary or secondary amine are preferably anhydrous if substantial contact with moisture and/or humidity during manufacturing, packaging, and/or storage is expected.

An anhydrous pharmaceutical composition should be prepared and stored such that its anhydrous nature is maintained. Accordingly, anhydrous compositions are preferably packaged using materials known to prevent exposure to water such that they can be included in suitable formulary kits. Examples of

suitable packaging include, but are not limited to, hermetically sealed foils, plastics, unit dose containers (e.g., vials) with or without desiccants, blister packs, and strip packs.

2.5.2 Controlled and Delayed Release Dosage Forms

Pharmaceutically acceptable salts of a CXCR4 antagonist can be administered by controlled- or delayed-release means. Controlled-release pharmaceutical products have a common goal of improving drug therapy over that achieved by their non-controlled release counterparts. Ideally, the use of an optimally designed controlled-release preparation in medical treatment is characterized by a minimum of drug substance being employed to cure or control the condition in a minimum amount of time. Advantages of controlled-release formulations include: 1) extended activity of the drug; 2) reduced dosage frequency; 3) increased patient compliance; 4) usage of less total drug; 5) reduction in local or systemic side effects; 6) minimization of drug accumulation; 7) reduction in blood level fluctuations; 8) improvement in efficacy of treatment; 9) reduction of potentiation or loss of drug activity; and 10) improvement in speed of control of diseases or conditions. Kim, Cherng-ju, Controlled Release Dosage Form Design, 2 (Technomic Publishing, Lancaster, Pa.: 2000).

Conventional dosage forms generally provide rapid or immediate drug release from the formulation. Depending on the pharmacology and pharmacokinetics of the drug, use of conventional dosage forms can lead to wide fluctuations in the concentrations of the drug in a patient's blood and other tissues. These fluctuations can impact a number of parameters, such as dose frequency, onset of action, duration of efficacy, maintenance of therapeutic blood

levels, toxicity, side effects, and the like. Advantageously, controlled-release formulations can be used to control a drug's onset of action, duration of action, plasma levels within the therapeutic window, and peak blood levels. In particular, controlled- or extended-release dosage forms or formulations can be used to ensure that the maximum effectiveness of a drug is achieved while minimizing potential adverse effects and safety concerns, which can occur both from under dosing a drug (i.e., going below the minimum therapeutic levels) as well as exceeding the toxicity level for the drug.

Most controlled-release formulations are designed to initially release an amount of drug (active ingredient) that promptly produces the desired therapeutic effect, and gradually and continually release other amounts of drug to maintain this level of therapeutic or prophylactic effect over an extended period of time. In order to maintain this constant level of drug in the body, the drug must be released from the dosage form at a rate that will replace the amount of drug being metabolized and excreted from the body. Controlled-release of an active ingredient can be stimulated by various conditions including, but not limited to, pH, ionic strength, osmotic pressure, temperature, enzymes, water, and other physiological conditions or compounds.

A variety of known controlled- or extended-release dosage forms, formulations, and devices can be adapted for use with the a CXCR4 antagonist salts and compositions of the disclosure. Examples include, but are not limited to, those described in U.S. Pat. Nos.: 3,845,770; 3,916,899; 3,536,809; 3,598,123; 4,008,719; 5674,533; 5,059,595; 5,591,767; 5,120,548; 5,073,543; 5,639,476; 5,354,556; 5,733,566; and 6,365,185 B1; each of which is incorporated herein by

reference. These dosage forms can be used to provide slow or controlled-release of one or more active ingredients using, for example, hydroxypropylmethyl cellulose, other polymer matrices, gels, permeable membranes, osmotic systems (such as OROS® (Alza Corporation, Mountain View, Calif. USA)), multilayer coatings, microparticles, liposomes, or microspheres or a combination thereof to provide the desired release profile in varying proportions. Additionally, ion exchange materials can be used to prepare immobilized, adsorbed salt forms of a CXCR4 antagonist and thus effect controlled delivery of the drug. Examples of specific anion exchangers include, but are not limited to, Duolite® A568 and Duolite® AP143 (Rohm&Haas, Spring House, Pa. USA).

One embodiment of the disclosure encompasses a unit dosage form which comprises a pharmaceutically acceptable salt of a CXCR4 antagonist (e.g., a sodium, potassium, or lithium salt), or a polymorph, solvate, hydrate, dehydrate, co-crystal, anhydrous, or amorphous form thereof, and one or more pharmaceutically acceptable excipients or diluents, wherein the pharmaceutical composition or dosage form is formulated for controlled-release. Specific dosage forms utilize an osmotic drug delivery system.

A particular and well-known osmotic drug delivery system is referred to as OROS® (Alza Corporation, Mountain View, Calif. USA). This technology can readily be adapted for the delivery of compounds and compositions of the disclosure. Various aspects of the technology are disclosed in U.S. Pat. Nos. 6,375,978 B1; 6,368,626 B1; 6,342,249 B1; 6,333,050 B2; 6,287,295 B1; 6,283,953 B1; 6,270,787 B1; 6,245,357 B1; and 6,132,420; each of which is incorporated herein by reference. Specific adaptations of OROS® that can be

used to administer compounds and compositions of the disclosure include, but are not limited to, the OROS® Push-Pull™, Delayed Push-Pull™, Multi-Layer Push-Pull™, and Push-Stick™ Systems, all of which are well known. See, e.g. worldwide website alza.com. Additional OROS® systems that can be used for the controlled oral delivery of compounds and compositions of the disclosure include OROS®-CT and L-OROS® ; see, Delivery Times, vol. 11, issue II (Alza Corporation).

Conventional OROS® oral dosage forms are made by compressing a drug powder (e.g., a CXCR4 antagonist salt) into a hard tablet, coating the tablet with cellulose derivatives to form a semi-permeable membrane, and then drilling an orifice in the coating (e.g., with a laser). Kim, Cherng-ju, Controlled Release Dosage Form Design, 231-238 (Technomic Publishing, Lancaster, Pa.: 2000). The advantage of such dosage forms is that the delivery rate of the drug is not influenced by physiological or experimental conditions. Even a drug with a pH-dependent solubility can be delivered at a constant rate regardless of the pH of the delivery medium. But because these advantages are provided by a build-up of osmotic pressure within the dosage form after administration, conventional OROS® drug delivery systems cannot be used to effectively delivery drugs with low water solubility. Because a CXCR4 antagonist salts and complexes of this disclosure (e.g., a CXCR4 antagonist sodium) are far more soluble in water than a CXCR4 antagonist itself, they are well suited for osmotic-based delivery to patients. This disclosure does, however, encompass the incorporation of a CXCR4 antagonist, and non-salt isomers and isomeric mixtures thereof, into OROS® dosage forms.

A specific dosage form of the compositions of the disclosure comprises: a wall defining a cavity, the wall having an exit orifice formed or formable therein and at least a portion of the wall being semipermeable; an expandable layer located within the cavity remote from the exit orifice and in fluid communication with the semipermeable portion of the wall; a dry or substantially dry state drug layer located within the cavity adjacent the exit orifice and in direct or indirect contacting relationship with the expandable layer; and a flow-promoting layer interposed between the inner surface of the wall and at least the external surface of the drug layer located within the cavity, wherein the drug layer comprises a salt of a CXCR4 antagonist, or a polymorph, solvate, hydrate, dehydrate, co-crystal, anhydrous, or amorphous form thereof. See U.S. Pat. No. 6,368,626, the entirety of which is incorporated herein by reference.

Another specific dosage form of the disclosure comprises: a wall defining a cavity, the wall having an exit orifice formed or formable therein and at least a portion of the wall being semipermeable; an expandable layer located within the cavity remote from the exit orifice and in fluid communication with the semipermeable portion of the wall; a drug layer located within the cavity adjacent the exit orifice and in direct or indirect contacting relationship with the expandable layer; the drug layer comprising a liquid, active agent formulation absorbed in porous particles, the porous particles being adapted to resist compaction forces sufficient to form a compacted drug layer without significant exudation of the liquid, active agent formulation, the dosage form optionally having a placebo layer between the exit orifice and the drug layer, wherein the active agent formulation comprises a salt of a CXCR4 antagonist, or a polymorph, solvate, hydrate,

dehydrate, co-crystal, anhydrous, or amorphous form thereof. See U.S. Pat. No. 6,342,249, the entirety of which is incorporated herein by reference.

2.5.3. Parenteral Dosage Forms

Parenteral dosage forms can be administered to patients by various routes, including, but not limited to, subcutaneous, intravenous (including bolus injection), intramuscular, and intraarterial. Since administration of parenteral dosage forms typically bypasses the patient's natural defenses against contaminants, parenteral dosage forms are preferably sterile or capable of being sterilized prior to administration to a patient. Examples of parenteral dosage forms include, but are not limited to, solutions ready for injection, dry products ready to be dissolved or suspended in a pharmaceutically acceptable vehicle for injection, suspensions ready for injection, and emulsions. In addition, controlled-release parenteral dosage forms can be prepared for administration of a patient, including, but not limited to, administration DUROS®-type dosage forms, and dose-dumping.

Suitable vehicles that can be used to provide parenteral dosage forms of the disclosure are well known to those skilled in the art. Examples include, without limitation: sterile water; Water for Injection USP; saline solution; glucose solution; aqueous vehicles such as but not limited to, Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, and Lactated Ringer's Injection; water-miscible vehicles such as, but not limited to, ethyl alcohol, polyethylene glycol, and propylene glycol; and non-aqueous vehicles such as, but not limited to, corn oil, cottonseed oil, peanut oil, sesame oil, ethyl oleate, isopropyl myristate, and benzyl benzoate.

Compounds that alter or modify the solubility of a pharmaceutically acceptable salt of a CXCR4 antagonist disclosed herein can also be incorporated into the parenteral dosage forms of the disclosure, including conventional and controlled-release parenteral dosage forms.

2.5.4. Topical, Transdermal And Mucosal Dosage Forms

Topical dosage forms of the disclosure include, but are not limited to, creams, lotions, ointments, gels, shampoos, sprays, aerosols, solutions, emulsions, and other forms know to one of skill in the art. See, e.g., Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing, Easton, Pa. (1990); and Introduction to Pharmaceutical Dosage Forms, 4th ed., Lea & Febiger, Philadelphia, Pa. (1985). For non-sprayable topical dosage forms, viscous to semi-solid or solid forms comprising a carrier or one or more excipients compatible with topical application and having a dynamic viscosity preferably greater than water are typically employed. Suitable formulations include, without limitation, solutions, suspensions, emulsions, creams, ointments, powders, liniments, salves, and the like, which are, if desired, sterilized or mixed with auxiliary agents (e.g., preservatives, stabilizers, wetting agents, buffers, or salts) for influencing various properties, such as, for example, osmotic pressure. Other suitable topical dosage forms include sprayable aerosol preparations wherein the active ingredient, preferably in combination with a solid or liquid inert carrier, is packaged in a mixture with a pressurized volatile (e.g., a gaseous propellant, such as freon), or in a squeeze bottle. Moisturizers or humectants can also be added to pharmaceutical compositions and dosage forms if desired. Examples of such additional ingredients are well known in the art. See, e.g., Remington's

Pharmaceutical Sciences, 18.sup.th Ed., Mack Publishing, Easton, Pa. (1990).

Transdermal and mucosal dosage forms of the compositions of the disclosure include, but are not limited to, ophthalmic solutions, patches, sprays, aerosols, creams, lotions, suppositories, ointments, gels, solutions, emulsions, suspensions, or other forms known to one of skill in the art. See, e.g., Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing, Easton, Pa. (1990); and Introduction to Pharmaceutical Dosage Forms, 4th Ed., Lea & Febiger, Philadelphia, Pa. (1985). Dosage forms suitable for treating mucosal tissues within the oral cavity can be formulated as mouthwashes, as oral gels, or as buccal patches. Additional transdermal dosage forms include "reservoir type" or "matrix type" patches, which can be applied to the skin and worn for a specific period of time to permit the penetration of a desired amount of active ingredient.

Examples of transdermal dosage forms and methods of administration that can be used to administer the active ingredient(s) of the disclosure include, but are not limited to, those disclosed in U.S. Pat. Nos.: 4,624,665; 4,655,767; 4,687,481; 4,797,284; 4,810,499; 4,834,978; 4,877,618; 4,880,633; 4,917,895; 4,927,687; 4,956,171; 5,035,894; 5,091,186; 5,163,899; 5,232,702; 5,234,690; 5,273,755; 5,273,756; 5,308,625; 5,356,632; 5,358,715; 5,372,579; 5,421,816; 5,466;465; 5,494,680; 5,505,958; 5,554,381; 5,560,922; 5,585,111; 5,656,285; 5,667,798; 5,698,217; 5,741,511; 5,747,783; 5,770,219; 5,814,599; 5,817,332; 5,833,647; 5,879,322; and 5,906,830, each of which are incorporated herein by reference in their entirety.

Suitable excipients (e.g., carriers and diluents) and other materials that can be used to provide transdermal and mucosal dosage forms encompassed by this

disclosure are well known to those skilled in the pharmaceutical arts, and depend on the particular tissue or organ to which a given pharmaceutical composition or dosage form will be applied. With that fact in mind, typical excipients include, but are not limited to water, acetone, ethanol, ethylene glycol, propylene glycol, butane-1,3-diol, isopropyl myristate, isopropyl palmitate, mineral oil, and mixtures thereof, to form dosage forms that are non-toxic and pharmaceutically acceptable.

Depending on the specific tissue to be treated, additional components may be used prior to, in conjunction with, or subsequent to treatment with pharmaceutically acceptable salts of a CXCR4 antagonist of the disclosure. For example, penetration enhancers can be used to assist in delivering the active ingredients to or across the tissue. Suitable penetration enhancers include, but are not limited to: acetone; various alcohols such as ethanol, oleyl, an tetrahydrofuryl; alkyl sulfoxides such as dimethyl sulfoxide; dimethyl acetamide; dimethyl formamide; polyethylene glycol; pyrrolidones such as polyvinylpyrrolidone; Kollidon grades (Povidone, Polyvidone); urea; and various water-soluble or insoluble sugar esters such as TWEEN 80 (polysorbate 80) and SPAN 60 (sorbitan monostearate).

The pH of a pharmaceutical composition or dosage form, or of the tissue to which the pharmaceutical composition or dosage form is applied, may also be adjusted to improve delivery of the active ingredient(s). Similarly, the polarity of a solvent carrier, its ionic strength, or tonicity can be adjusted to improve delivery. Compounds such as stearates can also be added to pharmaceutical compositions or dosage forms to advantageously alter the hydrophilicity or

lipophilicity of the active ingredient(s) so as to improve delivery. In this regard, stearates can serve as a lipid vehicle for the formulation, as an emulsifying agent or surfactant, and as a delivery-enhancing or penetration-enhancing agent. Different hydrates, dehydrates, co-crystals, solvates, polymorphs, anhydrous, or amorphous forms of the pharmaceutically acceptable salt of a CXCR4 antagonist can be used to further adjust the properties of the resulting composition.

2.6. Kits

Typically, active ingredients of the pharmaceutical compositions of the disclosure are preferably not administered to a patient at the same time or by the same route of administration. This disclosure therefore encompasses kits which, when used by the medical practitioner, can simplify the administration of appropriate amounts of active ingredients to a patient.

A typical kit comprises a unit dosage form of a pharmaceutically acceptable salt of a CXCR4 antagonist and a unit dosage form of a second pharmacologically active compound, such as anti-proliferative agent, or anti-cancer agent. In particular, the pharmaceutically acceptable salt of a CXCR4 antagonist is the sodium, lithium, or potassium salt, or a polymorph, solvate, hydrate, dehydrate, co-crystal, anhydrous, or amorphous form thereof. A kit may further comprise a device that can be used to administer the active ingredient. Examples of such devices include, but are not limited to, syringes, drip bags, patches, and inhalers.

Kits of the disclosure can further comprise pharmaceutically acceptable vehicles that can be used to administer one or more active ingredients. For example, if an active ingredient is provided in a solid form that must be reconstituted for parenteral administration, the kit can comprise a sealed container of a suitable vehicle in which the

active ingredient can be dissolved to form a particulate-free sterile solution that is suitable for parenteral administration. Examples of pharmaceutically acceptable vehicles include, but are not limited to: Water for Injection USP; aqueous vehicles such as, but not limited to, Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, and Lactated Ringer's Injection; water-miscible vehicles such as, but not limited to, ethyl alcohol, polyethylene glycol, and propylene glycol; and non-aqueous vehicles such as, but not limited to, corn oil, cottonseed oil, peanut oil, sesame oil, ethyl oleate, isopropyl myristate, and benzyl benzoate.

Other kits include reagents for the detection or quantification of cancer cells or cancer cell metastasis and include for example, a labeled CXCR4 peptide antagonist. A representative labeled CXCR4 peptide antagonist is biotinylated TN14003. The kit can also include steptavidin conjugated with a detectable label such as a fluorophore. It will be appreciated that buffers for maintaining pH, osmolality, and conditions for binding of the labeled antagonist to a sample can be included. Additional reagent materials can optionally be included in the kits such as microtiter plates and cover slips, etc.

3. Methods and Materials

Cell Culture. Human breast carcinoma cell lines, MDA-MB-231 (a gift of Z. Bhujwalla, Johns Hopkins University, Baltimore) and MDA-MB-435 (a gift of Lily Yang, Emory University, Atlanta) were cultured in 5% CO_2 at 37°C in RPMI-1600 (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Sigma), 50 U/ml of penicillin, and 50 µg/ml of streptomycin (Invitrogen, Carlsbad, CA). Human primary fibroblast cells 2091 (ATCC) were cultured in DMEM (Sigma), supplemented with 10% FBS and antibiotics.

Antagonist and Control Peptide Synthesis and Biotin Labeling. The

CXCR4 antagonist was synthesized by the Microchemical Core Facility at Emory University. A control peptide was created by randomly scrambling the amino acid sequence of CXCR4 antagonist while maintaining the disulfide bond to maintain the U-type structure of the antagonist (NH_2-KY-Nal-YR-$_D$K-Cit-RCRRP-Cit-C-amide). This control peptide does not bind to CXCR4 protein (data not shown). The CXCR4 antagonist was biotinylated by using an EZ-Link Sulfo-NHS-LC-Biotinylation kit (Pierce, Rockford, IL). A desalting column (Pierce) was used to remove unbound biotin and salts. The average number of biotins per CXCR4 antagonist was determined by 2-(4'-hydroxyazobenzene) benzoid acid (HABA) test. To determine the ratio of biotin to CXCR4 antagonist, 1 ml of avidin-HABA solution (Pierce) was added into a cuvette and the absorbency of avidin-HABA reagent was measured at 500nm.

Tumor Cell Invasion Assay. For an *in vitro* model system for metastasis, a matrigel invasion assay using a matrigel invasion chamber from BD Biocoat Cellware (San Jose, CA) was used. SDF-Iα (400 ng/ml, R & D Systems, Minneapolis, MM) was added to the bottom chamber to induce the invasion of MDA-MB-231 cells through the matrigel. CXCR4 antagonist (4 ng/ml) or anti-CXCR4 antibodies (MAB 173, R & D Systems) (25 ng/ml) were added to the cells before the cells were seeded to the top chamber. The matrigel invasion chamber was incubated for 22 hours in a humidified tissue culture incubator. First, non-invading cells were removed from the top of the matrigel with a cotton tipped swab. Invading cells at the bottom of the matrigel were fixed in methanol and stained with hematoxylin and eosin (H&E). The invasion rate was determined by counting the H&E stained cells.

Cytotoxicity. A Cell Proliferation Assay (Promega, Madison, WI) was used to determine the cytotoxicity of the CXCR4 antagonist *in vitro*. The cell proliferation was measured by the Cell Titer 96 AQ (Promega). Cells were seeded in 96 well clear plates (3000 cells per well in 100 µl of medium) with different concentrations of CXCR4 antagonist. Two days later, 20 µl of CellTiter 96AQ reagent was added into each well, incubated for additional two hours, and the absorbance at 490 nm was measured.

***In vitro* Hemopoietic Progenitor Cell Colony Formation.** CXCR4 antagonists were evaluated for the toxicity on haemopoietic progenitor cell colony formation. Human bone marrow cells were obtained from healthy adult volunteers by iliac crest puncture and aspiration into preservative-free heparin under a protocol approved by the University of Michigan's Investigational Review Board. Mononuclear cells were isolated by density separation on Ficoll-hypaque (specific gravity 1.077). Following two rounds of plastic adherence at 37°C for one hour each in IMDM with 10% fetal bovine serum, 10% equine serum, and 1 µM hydrocortisone (Invitrogen), the non-adherent cells were recovered. $CD34^+$ bone marrow cells were isolated by positive immunoselection from the low density non-adherent cell fractions (Miltenyi Biotec Inc., Auburn, CA). Thereafter, the cells were cultured in 35-mm Petri dishes (Stem Cell Technologies) in a 1.1-mL mixture of 0.8% methylcellulose in alpha medium (Invitrogen) supplemented with 30% PCS, 1% bovine serum albumin (BSA; Stem Cell Technologies), 10^{-4} β-ME, 5 U/mL human erythropoietin (hEpo; Janssen-Cilag), and 2% spleen cell-conditioned medium (SCCM; Stem Cell Technologies) in the presence or absence of 1.4 mg/mL G418 and 2 µg/mL doxycycline (Sigma). Colonies were scored on day 14 of

incubation as derived from colony-forming units - granulocyte/macrophage (CFU-GM), or burst-forming units - erythroid (BFU-Es). The identification of colonies was confirmed by Wright-Giemsa staining of cytospin preparations of colonies. CXCR4 antagonist was added everyday at ½ the dose following the initial dose at day 0. The drug is stable for at least 36 hours and decays with a half life of 20 hours in RPMI medium with 10% FBS inside of a CO2 tissue culture incubator (data not shown).

FACS analysis. MDA-MB-231 and MDA-MB-435 cells grown on 60 mm dish were incubated with 0.5 µg/ml of biotinylated CXCR4 antagonist for 20 min on ice. Following the incubation, cells were collected and washed with a phosphate buffered saline solution (PBS). Streptavidin-conjugated Phycoerythrin (PE) or FITC was applied at 1:100 dilution to the cells. The cells were incubated for 30 minutes at room temperature in the dart, followed by three washes of PBS. The cells were resuspended in 600 µl of PBS, filtered through 30µm pore size (VWR, Willard, OH), and analyzed by using Beckton Dickinson FACScan equipped with Cell Quest software. PE or FITC fluorescence was detected in FL2 channel (excitation 488nm/emission 575nm) or in FL1 channel (ex 488nm/ em 530nm), respectively.

Animal Experiments. Animal experiments were performed on 6 to 8-weeks-old CB-17 SCID female mice (Taconic Farms, Germantown, NY) with 7 animals/group. 17β-estradiol (60 day release, 0.72 mg; Innovative Research, Sarasota, FL) was inserted subcutaneously to all animals one day before the tumor cell injection. All animals were injected twice with MDA-MB-231 tumor cells (2×10^6) delivered through the tail vein, 6 days apart (day 0 and day 6). For the

treatment, animals were intravenously injected with either CXCR4 antagonist or its control peptide (this does not recognize CXCR4) twice weekly (100 ng/g body weight), from day 0, immediately before the first injection of tumor cells. Mice were sacrificed 55 days following tumor cell injections. Major organs were harvested in optimum cutting temperature (O.C.T., Fisher Scientific, Suwanee, GA) compound and frozen in liquid nitrogen for the presence of metastasis. The collected tissue sections were subjected to H&E histostaining, RT-PCR, and Real-time RT-PCR of human CXCR4.

For *in vivo* toxicity studies, mice were injected with the CXCR4 antagonist at 100 ng/g body weight twice weekly for 45 days. The control mice were treated with vehicle by the same protocol. Following the 45-day treatment, the mice were sacrificed. Their liver and kidney sections were subjected to the evaluation of toxicity by H&E staining. All protocols for animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Emory University.

Histostaining and Immunofluorescence. Animal organs were snap-frozen in O.C.T. in liquid nitrogen, sectioned, and fixed in ice-cold acetone and maintained at -80°C. The tissues were stained with H&E to evaluate the presence/absence of tumors. For immunofluorescence detection of CXCR4, the sections were washed in water and PBS, and blocked to eliminate non-specific binding (Avidin and Biotin Blocking Solution, Zymed Laboratories, Inc., San Francisco, CA). The slides were subsequently incubated for 45 min at room temperature (RT) with 0.05 |ng/ml of biotinylated CXCR4 antagonist. The slides were washed three times with PBS and incubated in streptavidin-R-Phycoerythrin (1:150 dilution) (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30

min at RT. Finally the slides were washed with PBS and mounted in an anti-fade mounting solution (Molecular Probes, Eugene, OR).

Formalin fixed paraffin embedded tissue sections were heated at 58°C for 30 min. These specimens were washed with xylene three times for 5 min each, followed by washes with 100%, 95%, and 75% ethanol and rinsed with PBS. To block non-specific binding, the samples were incubated in avidin-block and biotin-block sequentially. The biotinylated CXCR4 antagonist (0.05 jug/ml) was applied to tissue sections and the samples were further incubated for 45 min in a humidified chamber at room temperature. The slides were washed three times with PBS and incubated in streptavidin-Rhodamine (1:150 dilution) (Jackson ImmunoResearch Laboratories) for 30 min at RT. After washing and mounting in an anti-fade mounting solution (Molecular Probes), the samples were analyzed on a Nikon Eclipse E800 microscope. All protocols for human tissue studies were reviewed and approved by the Institutional Review Board (1KB) at Emory University.

Northern and Western Blot Analyses. For Northern blot analysis, total RNA (15 µg) was prepared with Trizol (Invitrogen) according to manufacturer's instruction and loaded on a 1.4% agarose-formaldehyde gel. After transferring to nitrocellulose, the blot was probed with ^{32}P-labeled CXCR4 fragments (Genbank Accession # AI920946) and later washed once in 2X SSC (IX SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.5% sodium dodecyl sulfate (SDS) for 30 min at RT, and three times in 0.2XSSC-0.5% SDS for 30 min at 50°C. For Western blot analysis, equivalent concentrations of total cellular proteins were resolved by SDS/PAGE (10% gel) and subjected to immunoblot analysis using polyclonal rabbit anti-CXCR4 antibody (Ab-2, Oncogene) and a monoclonal mouse anti-(3-actin

(Sigma).

RT-PCR and Real-time RT-PCR Analyses. For RT-PCR, total RNA was prepared from three slices of frozen tissues from animal organs with Trizol (Invitrogen), according to manufacturer's instruction. The human CXCR4-specific primers for 149 base pairs are 5'-GAACCCTGTTTCCGTGAAGA (SEQ ID NO: 17) and 5'-CTTGTCCGTCATGCTTCTCA (SEQ ID NO: 18) (Genbank Accession number NM_003467) and the primers for β-actin are 5'-GACAGGATGCAGAAGGAGAT (SEQ ID NO: 19) and 3'-TGCTTGCTGATCCACATCTG (SEQ ID NO: 20) (Genbank Accession number X00351). First strand cDNA synthesis was done using a GeneAmp Gold RNA PCR Reagent Kit (Applied Biosystems). The 20 µl of volume included 0.5 µg of RNA, 200 µM dNTPs, 2.5 mM $MgCl_2$, 10 mM DTT, 8 U RNase inhibitor, 30 U of reverse transcriptase and 1.25 µM of random hexamers in 1 x RT buffer. The reaction was performed at 42°C for 30 min followed by 25°C for 10 min. The reaction was stopped by heating the samples to 95°C for 10 min. The RT reaction was stored at -20°C until usage, or immediately used as a template for the PCR. The reaction of cDNA PCR was carried out in a 20-µl reaction volume containing 2 µl of 10X buffer, 0.2 (µM concentration of forward and reverse primers, 3 mM of $MgCl_2$, 200 µM of each dNTP, and 2 µl of cDNA, at 95°C for 3 min, followed by 35 cycles of 94°C for 30s, 52°C for 30s, and 70°C for 1 min. The PCR products were analyzed by 1% agarose gel electrophoresis. For quantitative PCR analysis, SYBR Green quantitative PCR amplifications were performed in an iCycler with a multi-color Real-time PCR detection system (Bio-Rad, Hercules, CA). The reactions were carried out in a 15-µl reaction volume containing 7.5 µl

of 2X SYBR Green PCR Master Mix (Applied Biosystems, Fosty City, CA), 0.2 μM of each forward and reverse primer, and 1 μl of cDNA from RT-reaction described above. The thermal profile for the Real-time PCR was 95°C for 10 minutes followed by 40 cycles of 95°C for 30s, 54°C for 20s, and 72°C for 30s. In each run, a dilution series of the standards for CXCR4 gene and p-actin gene were run along with the unknown samples of the lung tissues. The automatic data acquisition and subsequent data analysis was performed by using the iCycler Program after PCR amplification. The average copy number of CXCR4 gene was calculated per μg of total RNA.

For RT-PCR is siRNA experiments, total RNA was prepared from frozen tissue sections of animal lungs with Trizol (Invitrogen) according to manufacturer's instruction. The human HPRT-specific primers pairs are from the previous report[12], the human CXCR4-specific primers for 149 base pairs are 5'-GAACCCTGTTTCCGTGAAGA (SEQ ID NO: 17) and 5'-CTTGTCCGTCATGCTTCTCA (SEQ ID NO: 18) (Genbank Accession no. NM_003467), and the primers for β-actin are 5'-GACAGGATGCAGAAGGAGAT (SEQ ID NO: 19) and 3'-TGCTTGCTGATCCACATCTG (SEQ ID NO: 20) (Genbank Accession no. X00351). First strand cDNA synthesis and amplification of the cDNA were done using a GeneAmp Gold RNA PCR Reagent Kit (Applied Biosystems) following manufacturer's instruction. For Real-time quantitative PCR analysis, SYBR Green quantitative PCR amplifications (Applied Biosystems) were performed in an iCycler with a multi-color Real-time PCR detection system (Bio-Rad).

The construction of siRNAs and transfection. Two different siRNA

duplexes were designed (Genbank Accession no. NM_003467 which is incorporated by reference in its entirety). The cDNA-targeted region and the sequence of the siRNAs duplexes are as follows: ^{197}-AATAAAATCTTCCTGCCCACC-^{217} (SEQ ID NO: 4) for siRNA1, ^{529}AAGGAAGCTGTTGGCTGAAAA-^{549} (SEQ ID NO: 5) for siRNA2. The non-specific Control siRNA duplexes were purchased from Dhamacon Inc. with the same GC content as CXCR4 siRNAs (42%, D001206-10). The siRNAs were transfected into MD A-MB-231 cells using lipofectiamine2000 (Invitrogen) *in vitro*. Human breast carcinoma cell line, MD A-MB-231 (a gift of Z. Bhujwalla, Johns Hopkins University, Baltimore) was cultured in 5% CO_2 at 37°C in RPMI-1640 (Sigma) supplemented with 10% fetal bovine serum (FBS; Sigma), 50 U/ml of penicillin, and 50 µg/ml of streptomycin (Invitrogen).

Detection of siRNA Efficiency. To determine the efficiency of siRNA, at 48 hours post-transfection, the cells were collected to make total RNA and cell lysate to measure the mRNA levels and protein levels of CXCR4 respectively from the transfected cells. At the same time point, cells were also immunostained by the biotinylated CXCR4 antagonist for immunofluorescence to measure CXCR4 protein levels. See for example Liang, Z. et al. Inhibition of Breast Cancer metastasis by selective synthetic polypeptide against CXCR4 chemokine receptor. Cancer Res. 64(2003) which is incorporated by reference in its entirety.. The CXCR4 antagonist was synthesized by the Microchemical Core Facility at Emory University and biotinylated by using an EZ-Link Sulfo-NHS-LC-Biotinylation kit (Pierce Biotech.) following the manufacturer's instruction. For Northern blot analysis, total RNA was prepared with Trizol (Invitrogen) according

to manufacturer's instruction and 15 jag of total RNA was loaded on a 1.4% agarose-formaldehyde gel. After transferring to nitrocellulose, the blot was probed with ^{32}P-labeled CXCR4 fragments (Genbank Accession # AI920946). For Western blot analysis we followed a previous protocol[20]. Briefly, equivalent amount of total cellular proteins was resolved by SDS/PAGE (12% gel) and subjected to immunoblot analysis using polyclonal rabbit anti-CXCR4 antibody (Ab-2, EMD Biosciences) and a monoclonal mouse anti-β-actin (Sigma).

Tumor Cell Invasion Assay. A matrigel invasion chamber from BD Biocoat Cellware was used for the matrigel invasion assay. The cells were added to the top chamber at 48 hours post-transfection. SDF-la (100 ng/ml, R & D Systems) was added to the bottom chamber to induce the invasion of MDA-MB-231 cells through the matrigel. After matrigel invasion chamber was incubated for 22 hours in a humidified tissue culture incubator, non-invading cells were removed from the top of the matrigel with a cotton tipped swab and invading cells at the bottom of the matrigel were fixed in methanol and stained with hematoxylin and eosin (H&E). The invasion rate was determined by counting the H&E stained cells.

Cytotoxicity. The cell proliferation was measured by the Cell Titer 96 AQ (Promega). This assay is similar to MTT assay based on cellular lactate dehydrogenase enzyme activity. The cells at 48 hours post-transfection with siRNAs of CXCR4 were seeded in 96 well clear plates (3000 cells per well in 100 pi of medium). Twenty-four hours later, 20 µl of CellTiter 96AQ reagent was added into each well and the plate was incubated for additional two hours. And then the absorbance at 490 nm was measured to determine the relative cell

numbers.

SiRNA Animal Experiments. Animal experiments were performed on 6 to 8-weeks-old CB-17 SCID female mice (Taconic Farms, Germantown, NY) with 6 mice/group. 17(3-estradiol (cat. no. SE-121, Innovative Research) was inserted subcutaneously to all animals one day before the tumor cell injection. All animals in the treated group were injected intravenously through the tail vein with 2×10^6 of MDA-MB-231 tumor cells transfected with CXCR4 siRNA1&2 at 48 hours prior to the injection and then were injected with CXCR4 siRNA1&2 twice weekly (0.5 µg/g body weight). For all animals in the control group were injected with 2×10^6 of MDA-MB-231 tumor cells with non-specific control siRNA duplexes and then were injected with control duplexes twice weekly. Mice were sacrificed at 45 days following tumor cell injection. The major organ tissues were harvested in optimum cutting temperature (O.C.T., Fisher Scientific) compound and frozen in liquid nitrogen. The frozen tissue sections were subjected to H&E histostaining and Real-time RT-PCR of the human housekeeping gene HPRT and CXCR4. All protocols for animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Emory University.

Statistical Analysis. All statistical significances were determined by Student's *t-test.*

EXAMPLES

Example 1: Specificity of CXCR4 antagonist

Initially experiments were performed to verify that the CXCR4 antagonist binds to the predicted SDF-1 binding sites on the CXCR4 receptor. For these studies, MDA-MB-231 cells were first incubated in the presence and absence of 2

μg/ml of SDF-1α for 10 min and then fixed in ice-cold acetone. Cells were immunostained by using biotin-labeled CXCR4 antagonist and streptavidin-conjugated rhodamine. The immunofluorescence of the biotin-labeled CXCR4 antagonist was negative in both membrane and cytosol in the cells pretreated with SDF-1α for 10 min (Figure 1A right). The utility of the biotinylated CXCR4 antagonist as a probe of CXCR4 coupled with immunofluorescence staining of paraffin-embedded tissues from breast cancer patients and cultured breast cancer cells was explored further. MDA-MB-231 had high levels of mRNA and protein for CXCR4 as shown in Northern blots and Western blots compared to MDA-MB-435 (Figure 1B). When the biotinylated CXCR4 antagonist was used to stain the two cell types, the high expressing MDA-MD-231 cells were brightly stained (Figure 1C left), consistent with the high protein levels of CXCR4. On the other hand, binding of biotinylated CXCR4 antagonist to MDA-MB-435 was dramatically less (Figure 1C right) consistent with the low surface CXCR4 expression in these cells. Flow cytometry was used to confirm these results, and demonstrated as expected that the MDA-MD-435 cells had limited binding of the biotinylated CXCR4 antagonist (Figure 1D top) in contrast to the results with MDA-MD-231 (Figure 1D bottom). Immunofluorescence staining with the biotinylated CXCR4 antagonist on cancer patients' paraffin-embedded tissue sections demonstrated that CXCR4 antagonist can be used to detect CXCR4 receptors of tumor cells from the archived paraffin-embedded tissue sections (Figure 1E). Figure IE shows that CXCR4 expression levels are low in normal tissues (no red rhodamine staining) while primary tumors and the lymph node metastasis from the same patient showed elevated CXCR4 protein levels. Importantly, many samples carrying the diagnoses of Ductal

Carcinoma *in situ* (DCIS) already acquired CXCR4 overexpression (Figure 1E).

Example 2: Inhibition of breast cancer cell invasion *in vitro* by CXCR4 antagonist

For an *in vitro* model system for metastasis, a matrigel invasion chamber (Becton & Dickinson, Franklin lakes, NJ) was used. SDF-1α was added to the lower chamber to attract CXCR4-positive breast cancer cells to migrate through the matrigel. In the absence of SDF-1, the invasion rate was very low. With 400 ng/ml of SDF-la in the bottom chamber, significantly greater numbers of MDA-MB-231 cells responded to the chemoattractant and migrated into the bottom chamber. This SDF-1 mediated invasion was suppressed by the addition of 25 μg/ml of antibody directed against the CXCR4 receptor (MAS 173, R & D) (Figure 2). Like the CXCR4 antibody, CXCR4 antagonist also inhibited invasion of the tumor cells *in vitro,* but did so more effectively at 4 ng/ml (2 μM) concentration. On the other hand, CXCR4-negative MDA-MB-435 cells failed to invade through matrigel even with SDF-la in the bottom chamber (right hand side of Figure 2).

Example 3: CXCR4 antagonist blocked breast cancer metastasis in animal model

To extend our in vitro findings an experimental metastasis animal model of breast cancer metastasis was established. MDA-MB-231 cells in conjunction with the control peptide or CXCR4 antagonist were administered twice intravenously to female SCID mice supplemented with 17β-estradiol. The CXCR4 antagonist or control peptide treatment was continued twice weekly for 55 days. All seven mice of the control group injected with MDA-MB-231 cells that were treated with control peptide developed lung metastases. Three representative pictures of lungs in figure 3 A exhibit bubble-looking lung micrometastasis in the control group (top panel). On the

other hand, three out of seven mice treated with CXCR4 antagonist i.v. twice weekly failed to form metastasis while four animals developed significantly smaller metastasis than the control peptide treated group. Three representative pictures of lungs in figure 3A show no visible lung metastasis in the group treated with CXCR4 antagonist (bottom panel). The tissues from the lung of these animals were processed for H&E staining. While the lung tissues from the CXCR4 antagonist-treated animals maintained the morphology of normal lung tissues, those from the control group were filled with tumor cells with big nuclei. The results were further confirmed by semi-quantitative Real-Time RT-PCR using CXCR4 primers that are specific for human CXCR4 (Figure 3B). These results demonstrated that there was significant expression of human CXCR4 mRNA in the metastasis-infiltrated lungs of those animals that were injected with MDA-MB-231 cells and treated with the control peptide (Figure 3B). In contrast, the RT-PCR analyses confirmed that there were significantly fewer metastases in the lungs of CXCR4 antagonist-treated SCID mice that were injected with MDA-MB-231 cells. These results agreed well with H&E staining of lung tissues (Figure 3A). Further analysis revealed that the average mRNA copy number for human CXCR4 per μg of total RNA of CXCR4 antagonist treated animals' lung was 10.6% of those in the control peptide treated lungs. Paralleling these findings were the observations that the average body weight was higher in antagonist treated animals compared to control peptide treated animals (Figure 3C). Lung weight reflected the tumor burden of the animal (Figure 3D).

Example 4: Cytotoxicity of CXCR4 antagonist

Decreased metastasis to the lung in CXCR4 antagonist treated animals could be due to failure to metastasize or to the cytotoxicity of the treatment. To determine

the cytotoxicity of the CXCR4 antagonist, CXCR4-positive MDA-MB-231 cells were treated with different concentrations of the antagonist and the effects on proliferation was determined. The CXCR4 antagonist did not affect cell proliferation even at 10 nM concentration (Figure 4A). Thus, it is unlikely that CXCR4 antagonist treated animals could not form large lung metastasis due to the cytotoxic effect of CXCR4 antagonist on MDA-MB-231 cells.

Example 5: Systemic Toxicity

In order to evaluate the possibility for systemic toxicity of the CXCR4 antagonist, several organs were examined microscopically. Figure 4B shows representative H & E staining of liver and kidney tissues from mice treated either with a PBS injection or CXCR4 antagonist. In particular, no central necrosis was observed in the liver and no tubular necrosis in the kidney. H & E staining results demonstrate that there was no damage in the livers or kidneys of the representative mice of each group. The CXCR4 antagonist was evaluated for the toxicity on hemopoietic progenitor cell colony. Colony formation from $CD34^+$ cells was determined and scored as either burst forming units-erythroid (BFU-E), or colony-forming units - granulocyte/macrophage (CFU-GM). CXCR4 antagonist was added everyday at ½ of a loading dose following the initial dose at day 0. For these studies, at 10 nM, the highest concentration tested, there was no discernable effect on hemopoietic progenitor cell colony formation (Figure 4C). The number of colonies per well was not significantly different with treatment for CFU-GM, BFU-E. Nor was the total number of colonies altered by addition of CXCR4 antagonist. Similar experiments were performed on human CXCR4-negative 2091 human primary fibroblast cells. Here too CXCR4 antagonist did not affect cell growth rate of 2091 cells,

even at 100 μM (50,000 times of its effective concentration) (Figure 4D).

Example 6: CXCR4 expression of MDA-MB-231 cells transfected by siRNAs of CXCR4

SiRNA transfected MDA-MB-231 cells were detected using the biotinylated CXCR4 peptide and streptavidin-phycoerythrin (PE). Red: CXCR4 PE staining; Blue: nuclei counter staining. SiRNA1 was more efficient in lowering CXCR4 expression than siRNA2, and the combination of siRNA1 and siRNA2 (SiRNA1+2) was more effective in lowering CXCR4 levels than either one alone (Fig. 5A). In Fig. 1B Western blot results of siRNA transfected MDA-MB-231 cells by using anti-CXCR4 antibody Ab2 (1:1000) show that SiRNA1+2 blocked the expression of CXCR4 protein almost completely. β-actin (Sigma, 1:2500) was used as a loading control. In Fig. 1C RT-PCT analysis of CXCR4 in siRNA transfected MDA-MB-231 cells show siRNA1+2 effectively blocked the expression of CXCR4.

The combination of two siRNAs of CXCR4 achieved almost complete suppression of CXCR4 expression in MDA-MB-231 cells at 48 hours post-transfection at both mRNA and protein level. The siRNA1 was more efficient in lowering CXCR4 expression than siRNA2. The combination of siRNA 1 and siRNA2 (siRNA 1+2) was more effective at suppressing CXCR4 than either one alone. The results demonstrated that siRNAs are efficient of inhibiting CXCR4 gene and protein expression.

Example 7: Invasion rates of MDA-MB-231 cells transfected with siRNAs of CXCR4

The effect of CXCR4 inhibition on invasion was determined by the matrigel invasion assay. The cell surface expression of CXCR4 allows a response to

SDF-1, resulting in increased migration and invasiveness. The CXCR4 ligand, SDF-1 (100 ng/ml) was added to the lower chamber to attract CXCR4-positive breast cancer cells to migrate through the matrigel. The invasion between control MDA-MB-231 breast cancer cells (transfected with non-specific Control siRNA duplexes) and siRNAs of CXCR4 transfected MDA-MB-231 cells were compared at 48 hours post-transfection. The invasion rate decreased to 39% of the control cells when cells were transfected with siRNA1, to 51% with siRNA2, and only to 16% with siRNA1+2 (Fig. 6). The data demonstrated an enhanced gene silencing with a combination of two different siRNAs not only at the protein or mRNA expression level, but also at the functional level. The invasion rates of MDA-MB-231 cells transfected with SiRNA1&2, SiRNA1, and siRNA2 relatively to the control are 6.9% (P=0.00028), 35.6% (P=0.00140), and 51.5%(P=0.00255) respectively.

Example 8: The effect of siRNAs of CXCR4 in breast cancer metastasis in an animal model

Fig. 7A is a photograph of the whole lungs of three mice from each group, and H&E staining of these lung tissues show that the lungs from the treated group mice were normal while the lungs from the control group mice were filled with human tumor cells. Fig. 7B shows real-time RT-PCR of hHPRT results of lung samples from all animals in each group. Number 1-6 are the lung tissue samples from 6 animals of the siRNA-treated group and Number 7-12 are those from 6 mice of the control group. The percentage of hHPRT expression level is relative to the calibrator sample number 1 from control group. Only two of six lungs from the siRNA of CXCR4 treated group mice expressed detectable hHPRT.

MDA-MB-231 cells transfected with SiRNA1+2 of CXCR4 were

administered once intravenously to female SCID mice supplemented with 17β-estradiol. 17β-estradiol increases the efficiency of tumor formation in both hormone-dependent and independent cell lines in animal. The synthetic siRNA-mediated RNA interference in human cells is transitory with cells recovering from a single treatment in four to six days. Therefore, in order to maintain the silencing effect of CXCR4 gene, the silencing siRNAs was introduced periodically. Treated mice formed very few metastases in their lungs in 45 days. The control group injected with control MDA-MB-231 cells developed lung metastases. Representative pictures of lungs in Fig. 7A show lungs exhibiting bubble-looking lung micrometastases in the control group. On the other hand, three representative pictures of lungs show no visible lung metastases in the group treated with siRNAs of CXCR4 (Fig. 7A). The H&E stainings of the lung tissues from the siRNA-treated animals show the morphology of normal lung, while those from the control group show the invading tumor cells (Fig. 7A). These results were further confirmed by semi-quantitative Real-time RT-PCR using human housekeeping gene HPRT primers (Fig. 7B). The lung metastasis was detected by H&E staining and RT-PCR of human housekeeping gene, hHPRT that does not cross-react with its mouse counterpart. hHPRT was used to detect CXCR4-siRNA transfected MDA-MB-231 cells because CXCR4 levels in these cells could not represent the presence of metastasis in the lungs.

Real-time RT-PCR analyses confirmed that there was high expression of human HPRT mRNA in metastasis-infiltrated lung of the SCID mice injected with the control MDA-MB-231 cells. In contrast, there were fewer metastases in lungs of the treated-siRNA SCID mice. In this group only two out of six mice were

observed weak expression of hHPRT gene (Fig. 7B). Real-time RT-PCR for human specific CXCR4 gene of these lung tissues demonstrated that high CXCR4 expression was observed in the control group mice lungs while very low CXCR4 expression in the lungs of the treated group mice (2.3 ± 2.2% of the control group). These results demonstrated that MDA-MB-231 cells with lowered CXCR4 levels did not form metastasis in our animal model. Decreased metastasis to the lung in CXCR4 siRNA treated animals could be due to failure to metastasize or to the cytotoxicity of the siRNA of CXCR4. The potential cytotoxicity of the siRNA of CXCR4 was measured by using Cell Titer AQ96 Assay kit (Promega) that is similar to MTT assay on those reseeded cells transfected with either non-specific Control siRNAs or the siRNAs of CXCR4 after 48 hour post-transfection. The growth of SiRNA1+2 transfected cells was 85.9 ± 15.7 % (p = 0.139) of that of control cells over 24 hours, indicating a relatively minor effect of siRNA treatment on cell growth. It is unlikely that the treated animals did not form lung metastases due to the cytotoxic effect of siRNA of CXCR4 on MDA-MB-231 cells. This lack of cytotoxicity also implies a limited systemic effect on normal cells.